

PLANT GROWTH AND DEVELOPMENT

Hormones and Environment



Lalit M. Srivastava

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Preface

This book deals with plant growth and development as regulated by hormones and environment. It is organized into five sections: I. Some Special Aspects of Plant Growth and Development; II. Structure and Metabolism of Plant Hormones; III. Hormonal Regulation of Developmental and Physiological Processes; IV. Molecular Basis of Hormone Action; and V. Environmental Regulation of Plant Growth. Each section has its own minipreface, which explains the content and rationale for that section. Plant growth and development is a vast topic. This book deals only with the better known or better studied aspects of plant growth regulation by hormones and by light and gravity among environmental factors. Areas not covered include flowering as regulated by daylength, the biological clock, and temperature; perception of environmental factors such as temperature, UV radiation, and enhanced CO₂ levels; and stresses caused by pests and pathogens.

The book is written for third and fourth year undergraduates and beginning graduate students who have had basic botany and some introduction to biochemistry and molecular biology. It is designed to complement texts in plant physiology. Like most other text books, it originated because there was no single text that covered the burgeoning field of hormonal and environmental regulation of plant growth that was reasonably up to date. The last text on this subject, "Growth and Differentiation in Plants," authored by P. F. Wareing and I. D. J. Phillips, was published in 1981. Since then, research in plant growth and development, spurred on by the application of molecular and genetic techniques, has revolutionized our thinking in plant biology.

This is not to say that there are no excellent books around. Several multiauthored books are available. Nevertheless, there is no text that is specifically written for senior undergraduates that covers the field of

hormonal and environmental regulation of plant growth.

This book has departed from the traditional texts in two important ways. A senior undergraduate needs to know not only what the results are but also how they were arrived. Accordingly, a good deal of emphasis in this book is on methodology, and illustrations often carry legends that provide information on the experimental protocol. The second departure is the manner in which the references appear. They are omitted from the body of the text for ease of reading, but are listed at the end of each chapter and arranged by topics following the headings and subheadings in each chapter.

Single-authored texts at the undergraduate level in this field are becoming a rarity, partly because the subject is so vast and growing so rapidly that it is difficult for any individual to keep abreast of the new and sometimes highly specialized information. Yet single-authored books offer a few advantages over multiauthored volumes—the text is closely integrated and there is a uniformity of presentation. The danger is that the chances of errors of fact and interpretation are multiplied. To keep these errors to a minimum, I have relied heavily on critical reading of each chapter, or sections or subsections of a chapter, by colleagues who are experts in that field. Without the help and critical advice of these colleagues, this book could not have been written. A list of these reviewers and contributors follows this preface. Two individuals deserve special thanks: Tom Berleth at the University of Toronto and Ravindar Kaur Sawhney at Yale University, for contributing information for a Box each. We truly live in a global village and the advent of internet and e-mail has made communication not only rapid, but also personal and informative. Numerous other colleagues around the globe shared their knowledge on specific points and/or provided illustrations. I would like to

express my deep appreciation to all these individuals for giving their time and sharing their knowledge. If the book is well received by students and instructors, credit goes in a very large measure to these individuals. Any errors or omissions are mine.

Many other individuals played important roles in completion of this book. They include Alex English, a former SFU undergraduate, and Nathalie Matheus, a graduate student, who read many sections of the book and offered valuable advice; Kerry Griffin and Derek Vance Steel who provided expert word processing help; and Tracy Lee who provided secretarial help with a smile. I am indebted to the staff at the Instruc-

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I

SOME SPECIAL ASPECTS OF PLANT GROWTH AND DEVELOPMENT

The chapters in Section I of this book are designed to serve two purposes. First, to highlight the fact that plants, while sharing many building blocks and metabolic pathways with animals, nonetheless are organized along different lines and have adopted different strategies for survival. Second, the chapters provide a backdrop for topics covered in Sections III and V, which deal with hormonal and environmental regulation of plant growth. Chapter 1 describes the basic organization and development of an angiospermous plant. Chapter 2 is devoted to a discussion of the structure of cell wall and its importance, the major features of cell cycle and cell division in plants, and types of cell growth. Chapter 3 highlights patterning in zygotic embryo development and discusses somatic embryogenesis, an ability that plants have as a result of open growth and open differentiation. Chapter 4 deals with the question of determination, differentiation, and dedifferentiation in plants in the context of the inherent plasticity of plant development. An Appendix on molecular and genetic techniques used for the study of plant development concludes Section I.

1

Special Features of Plant Development

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1. PLANTS HAVE EVOLVED SOME NOVEL STRATEGIES FOR SURVIVAL

Plants are rooted to soil and hence have evolved several strategies for survival that are absent or unheard of in animal life. Nowhere are these strategies

more clearly evident than in the manner in which the plants grow and their cells and tissues differentiate.

1.1. Plants Show an Open Form of Growth

Plants are unique multicellular organisms that retain the capacity for unlimited growth, that is an overall increase in size, throughout their lives. They are able to do so because they have **meristems** at certain locations in the body. Meristems are composed of stem cells, which perpetuate themselves by cell divisions and also give rise to derivative cells, which differentiate along new lines. As a result of meristematic activity, fresh quotas of tissues and organs are formed, and the plant continues to grow in height and, in many cases, girth throughout its life. This form of growth in plants is referred to as an **open form of growth**. The advantages to this type of growth for organisms that are rooted but subject to predation are immediately obvious.

In contrast, in most animals the adult life is characterized by a cessation of growth, except in some instances where regeneration of an organ or body part occurs. While overall growth stops in animals, cell divisions and a turnover of cell populations may continue in stem cells at several locations and add new derivatives. In contrast, in plants, a turnover of cells in mature tissues is rare.

Plant meristems are classified on the basis of location (Fig. 1-1). **Apical meristems** are located at or near the tip of shoot or root and are called shoot and root apical meristems; they are responsible for primary growth, including elongation growth, of these organs. **Lateral meristems**, e.g., vascular cambium, cork cambium, are

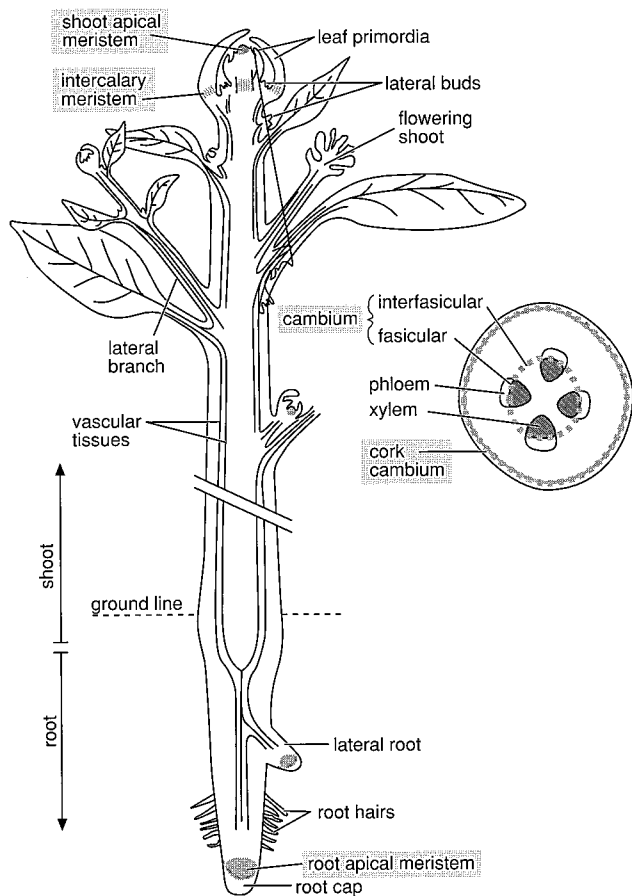


FIGURE 1-1 Diagrammatic representations of a dicot plant showing the principal meristems. Shoot and root apical meristems are indeterminate meristems—they continue to produce new shoot and root tissues throughout the life of the plant. In contrast, leaf and flower development involves meristems that function only for a defined duration—they are examples of determinate meristems. Intercalary meristems typically occur in grass stems and leaves, but are shown here to denote their locations. In dicots and gymnosperms, a vascular cambium originates in parts that have ceased elongation. In stems, it originates between primary xylem and primary phloem in a vascular bundle, or fascicle, and extends to the interfascicular areas, thus establishing a complete cambial ring. In roots, it arises slightly differently, but eventually forms a complete ring. In older roots and stems, the epidermis cannot keep pace with increasing girth and is replaced by a new tissue called periderm. Periderm owes its origin to a new meristem, called cork cambium or phellogen.

located on the sides of roots or stems and add to the girth, or secondary growth, of these organs. **Intercalary meristems** typically occur intercalated between mature regions, as at the bases of grass leaves; however, in a broader sense, they can be considered to occur at the bases of leaf primordia and above the nodes in stems of nearly all plants and add to elongation growth of these organs. Meristems are also classified as **indeterminate** and **determinate**. Shoot and root apical meristems (although not necessarily the same meristem), and vascu-

lar cambium, remain active for the life of the plant; hence, they are referred to as indeterminate meristems. In contrast, meristems involved in leaf and flower development are active only for a short time and are used up in the formation of those organs; in some texts, they are referred to as determinate meristems.

1.2. Structural Support for the Plant Body Is Provided by Cell Walls

Plants absorb water and minerals from soil, CO_2 and O_2 from air, carry on photosynthesis, transport photoassimilates to growing parts where they are used for growth, store photoassimilates as food reserves (carbohydrates, proteins, lipids), reproduce, and eventually die. Because they are rooted, they have to grow continuously to search for new sources of water and minerals as well as compete against neighbors for light. Root and shoot apical meristems provide for this growth. In some cases, because of the activity of the apical and lateral meristems, they grow to phenomenal sizes, e.g., redwoods, eucalyptus, and banyan tree, and live to be 3000–4000 years old, e.g., bristle cone pine. Structural support for the plant body is made possible by the presence of cell walls. In young organs, turgor pressure inside cells also contributes to structural support. The presence of a cell wall is a mechanical necessity for plants because it provides rigidity and strength while allowing flexibility, but it also imposes major restrictions to cell growth. A study of the chemical composition and architecture of cell walls, therefore, is essential for understanding both cell division and cell growth in plants (see Chapter 2).

1.3. Plants Development Is Highly Plastic

Being rooted, plants have evolved to perceive environmental factors, such as light, gravity, temperature, water, and touch. These environmental stimuli are perceived, sometimes with exquisite precision, and a response is affected in terms of growth, differentiation, reproduction, and so on. Plant development, as in all organisms, is basically regulated by its genetic complement, but, in contrast to multicellular animals, it is also characterized by extreme plasticity. **Plasticity** is a term used to describe the ability to change form or shape in response to a change in environment; no genetic change is involved. This is illustrated by two examples.

Many aquatic or semiaquatic plants show the phenomenon of heterophylly, which is the production of two or more forms of leaves within the same individual. The buttercup, yellow water crowfoot (*Ranunculus flabellaris*), produces leaves that have three broad lobes,

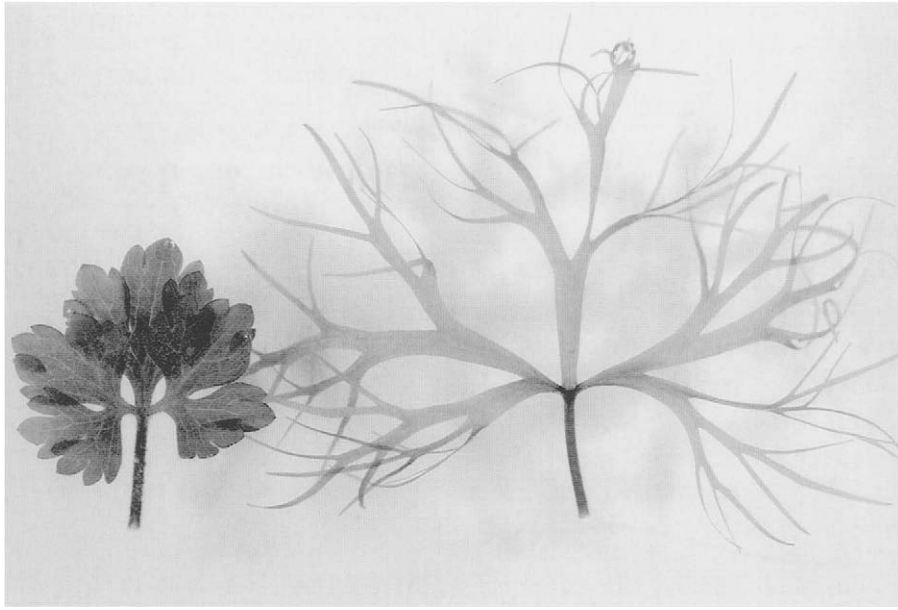


FIGURE 1-2 Heterophylly in buttercup, *Ranunculus flabellaris*. A leaf produced while the shoot apex was in air (left) and while it was under water (right). Courtesy of Michelle Woodvine and Nancy Dengler, University of Toronto, Toronto.

each lobe with one deep and several shallow sinuses, when exposed to air, but when submerged produces leaves that are highly dissected and filamentous (Fig. 1-2). The change in leaf form extends to changes in leaf anatomy and stomata. It should be noted that it is only the new leaves that are produced at the shoot apex subsequent to submergence that are dissected and filamentous—leaves that were produced before submergence do not change shape.

Seedlings of most dicots grown in the dark have long, spindly, yellow stems with small enation-like leaves at nodes, whereas those grown in light have robust, although shorter, green stems and well-expanded leaves. The change from a dark-grown to a light-grown phenotype is dramatic and affects not only internode length, leaf form, and chlorophyll synthesis, but also many other features.

Except for light, the perception of environmental signals, or receptors for environmental signals, are not always clear or known; in fact, very little is known about them, but the signaling pathway in many cases involves hormones.

1.4. Plants Show Open Differentiation

Plant cells and tissues differentiate to perform specific functions, but under certain circumstances they retain the ability to revert to an earlier state and go off in a new direction. This ability is referred to as open differentiation and is dealt with in Section 5 of this

chapter. The plasticity of plant growth is possible because of both open growth and open differentiation.

2. GROWTH, DIFFERENTIATION, AND MORPHOGENESIS

Growth of an organism is defined as an irreversible increase in mass. Because mass is related to cell volume and cell number, growth refers to an irreversible increase in cell size (enlargement) or to an increase in cell size as well as cell number (cell division). Cell division, by itself, is not sufficient to result in growth. **Differentiation**, in contrast, refers to the acquisition of qualitative differences among cells of common ancestry, i.e., those derived from a cell or group of cells. It is by differentiation that cells in an organ or tissue become different from each other, or specialized for different functions, e.g., the epidermis, or mesophyll, or xylem or phloem cells in a leaf. From a functional viewpoint, differentiation is equivalent to specialization. **Morphogenesis** is the acquisition of form, how a plant or organ acquires its distinctive shape or form. Because plant cells, generally, are fixed in relation to each other and because they are cemented by the cell wall (they are not free to move about as in animal development), morphogenesis in plants is essentially a function of planes of cell divisions and direction of cell growth. The control of these two processes, therefore, is central to a study of plant morphogenesis.

The basic features of cell division and cell growth are dealt with in Chapter 2, following a discussion of the composition and structure of the cell wall. Hormonal regulation of cell division and cell growth is covered in Chapter 15.

3. ORGANIZATION OF THE PLANT BODY

3.1. Embryo Development in Angiosperms

All multicellular plants arise from a single cell, the fertilized egg or **zygote**. A series of cell divisions in the zygote, followed by differentiation, set out the pattern for the embryo, which, if undisturbed, lasts throughout the life of the plant.

Embryo development in vascular plants, which include club mosses, horsetails, ferns, gymnosperms, and angiosperms, varies considerably. This section considers the typical embryo development in angiosperms. For other groups of vascular plants and for variations among angiosperms, the reader is referred to the excellent texts by Steeves and Sussex (1989) and Johri *et al.* (1992).

In angiosperms, the first division of the zygote is often, although not always, asymmetric and sets up an **apical** cell toward the chalazal end and a **basal** cell toward the micropylar end of the ovule (Fig. 1-3). The apical cell is smaller and densely cytoplasmic, whereas the basal cell is larger and has a big vacuole. These are only the most obvious differences between these two cells; at the cytoskeletal and especially biochemical level, there must be many other differences, although they still need elucidation. The fates of these two cells are dramatically different.

Divisions in the apical cell give rise to the embryo proper, which goes through several, but continuous, developmental stages, an eight-celled **proembryo** is followed by **globular**, **heart-shaped**, and **torpedo** stages (Fig. 1-3). The body plan of the embryo, i.e., the setting out of the **root** and **shoot poles**, **root-shoot axis**, and **cotyledons**, is evident between heart and torpedo stages. "Relatively uncommitted" (see Section 4.2.1 in this chapter; also, Chapter 4) groups of cells at the root and shoot poles become the future **root** and **shoot apices** with their own distinctive organizations. Embryonic tissues are also distinguished very early. A **protoderm** is evident already in the globular embryo, and the **ground meristem** and **procambium** become distinguished in the heart-shaped embryo. Further growth of the embryo continues for some time by cell division and cell enlargement, but eventually growth ceases and the embryo enters a

period of quiescence; further growth occurs only after germination. In many cases, because of the limited space within the ovule, now the seed, the embryo bends over on itself.

The basal cell divides to give rise to a structure known as the **suspensor**, which varies in size, from small and filamentous to massive, and which is believed to anchor the embryo in its proper position in the developing seed. The development of the suspensor generally precedes that of the embryo proper, and there is evidence that the suspensor serves to provide nutrition and hormones to the very young embryo. Some of these substances may be transported from the mother plant, whereas others are synthesized *in situ* in suspensor cells (see also Chapter 18). After the globular stage, the suspensor often shrivels up and becomes nonfunctional. While most of the suspensor is distinct from the embryo proper, the part proximal to the embryo, the **hypophysis**, contributes to the root apex.

The description just given pertains to most dicot embryos, but there are exceptions. For example, the setting out of the embryo proper and the suspensor is not always traceable to the first division of the zygote, although it occurs early on. Also, the subsequent divisions may not be as regular as those described, although ultimately the major organs and tissues are formed in expected places.

The embryo development in dicots and monocots is similar to the globular stage, but later in dicots the shoot apex develops along the same axis as the root apex and the two cotyledons occur laterally; whereas in monocots, the shoot apex occupies a lateral position and the single cotyledon is more terminal (Fig. 1-3k). This seemingly minor difference in embryo development between these two classes of flowering plants has enormous taxonomic significance and became established very early in the origin of flowering plants.

How do apical and basal cells come to have divergent destinies? What is the basis for the orderly unfolding of the embryonic pattern? How are the root-shoot polarities established? The answers to these questions are still largely unknown. However, application of molecular genetic techniques to analysis of pattern formation, improvements in techniques of isolating very young embryos and their *in vitro* culture, isolation of embryo sac and egg cell and *in vitro* fertilization, and mass production of somatic embryos at nearly synchronous stages hold great promise that environmental factors, including maternal signals, if any, that set up these patterns and polarities will soon be revealed.

We will go into some of this literature in Chapter 3. This section continues with organization of the adult plant body.

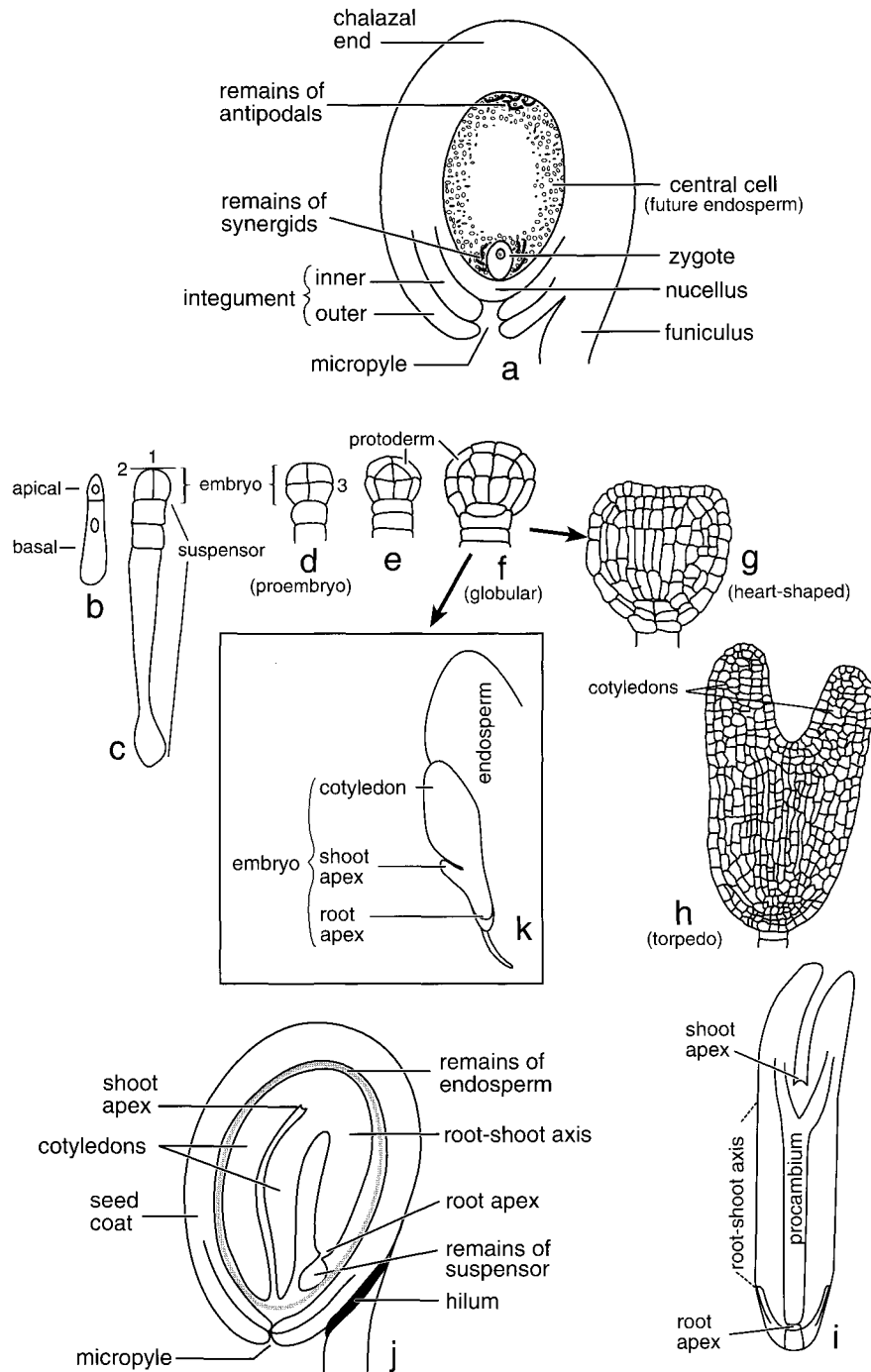


FIGURE 1-3 Stages in embryo development in a typical angiosperm. (a) An ovule after fertilization with the inner and outer integuments (which later form the seed coat), the micropyle, the chalazal end, and the central cell (formerly the embryo sac) with the zygote and several endosperm nuclei. The ovule is attached to the placenta in the ovary *via* a funiculus. (b and c) The first division of the zygote sets out apical (toward the chalaza) and basal (toward the micropyle) cells, which give rise to the embryo proper and the suspensor, respectively. The first three divisions in the apical cell, which give rise to the eight-celled proembryo, are numbered. (d–h) The embryo goes through several developmental stages, an eight-celled proembryo (d), globular (e and f), heart-shaped (g), and torpedo (h) stages. A protoderm is evident in the globular embryo, and the ground meristem and procambium become distinguished in the heart-shaped embryo. Cotyledons and a root–shoot axis become evident between heart and torpedo stages. The suspensor is not shown in these stages, it usually degenerates by the globular stage embryo. (i and j) Enlargement of the embryo continues for some more time, but eventually ceases; further growth is resumed only after germination. Because of the limited space, the embryo in some cases bends

3.2. The Adult Body

3.2.1. The Root Apex

The root and shoot poles established in the transition from heart- to torpedo-shaped embryo become organized as the root and shoot apices with their own distinctive structure, patterns of division, and the nature of derivatives. The **root apex** is responsible for the orderly growth of roots, whereas the **shoot apex** is responsible for the orderly growth of stems, as well as the initiation of new leaves, branches, and flowers.

The two apices are organized along different lines (cf. Figs. 1-4 and 1-8A). In most roots, the root apical meristem is not terminal but subterminal and is covered over by a structure known as the **root cap** (Fig. 1-4). The root cap serves some very useful functions for the plant. It is important in the perception of gravity such that roots grow toward the earth. It protects the apical

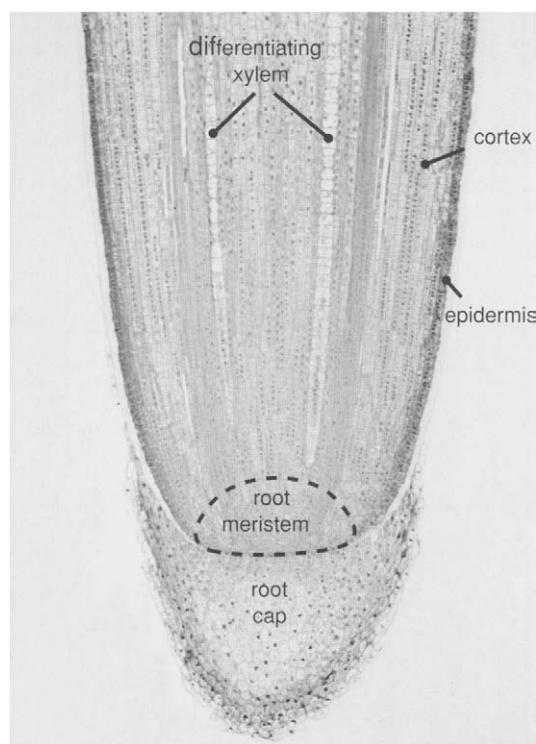


FIGURE 1-4 Longitudinal section of a maize (*Zea mays*) root showing the root apical meristem and the root cap. The section passes through parts of two longitudinal files of differentiating xylem cells.

meristem from abrasion during growth through the soil. The outer cells of the root cap keep getting sloughed off as new cells are added from within by the root apical meristem. It also secretes various types of compounds; mucilage, which facilitates the passage of root through the soil, and other carbohydrates and phenolics, which are important in interactions of plant root with microbes and fungi in the rhizosphere.

Cell divisions occur throughout the apical meristem, although a group of cells in the middle, the so-called **quiescent center**, divides much less frequently than others at the periphery. This can be shown by feeding radioactive thymidine to growing roots. The radioactive thymidine gets incorporated into DNA of dividing cells and the radioactivity can be visualized on an X-ray film (Fig. 1-5A). For quantitative measurements, the frequency of cell divisions in different parts of the root apex is calculated by counting the number of mitotic figures in sections of roots sampled over a defined time interval, but this a more laborious process (Fig. 1-5B).

The divisions in the apical meristem add new cells both acropetally to the root cap and basipetally to the zone of elongation and differentiation. In this latter zone, component cells continue to divide; they elongate as well, such that the whole organ elongates. Also, various tissue types, epidermis, cortex, endodermis, xylem, and phloem, begin to differentiate (Fig. 1-6). As a result of this elongation, the root tip is constantly pushed downward into the soil. Further basipetally, elongation gradually comes to a stop, whereas individual cell and tissue types, especially xylem elements, complete their differentiation and mature. In this zone of maturation, certain epidermal cells give rise to **root hairs**, which enhance the surface area of epidermal cells several fold and thus help in the absorption of water and minerals from the soil. **Endodermal cells**, with their specialized Casparian strip (or band), are involved in selective screening in the uptake of minerals and are well differentiated in the root hair zone.

In the region that is no longer involved in extension (or elongation) growth, **lateral roots** arise by localized divisions in the pericycle (Fig. 1-7). The nascent root primordium (primordium = a developing unit) grows through the cortex and epidermis of the parent root to emerge as a new lateral root. Lateral roots have a similar organization as the parent root and serve likewise for

over on itself. This period of enlargement is accompanied by reserve food deposition. In some seeds, the endosperm gets used up during this period, and the food reserves are deposited in the cotyledons and the root-shoot axis (see j); in others, the endosperm persists and is the main site of food deposition. (k) Embryo development in monocots is similar to that in dicots to the globular stage. Subsequently, in monocots, the shoot pole arises not in a straight line with the root pole, but laterally, and the single cotyledon occupies a more terminal position. Adapted from West and Harada (1993).

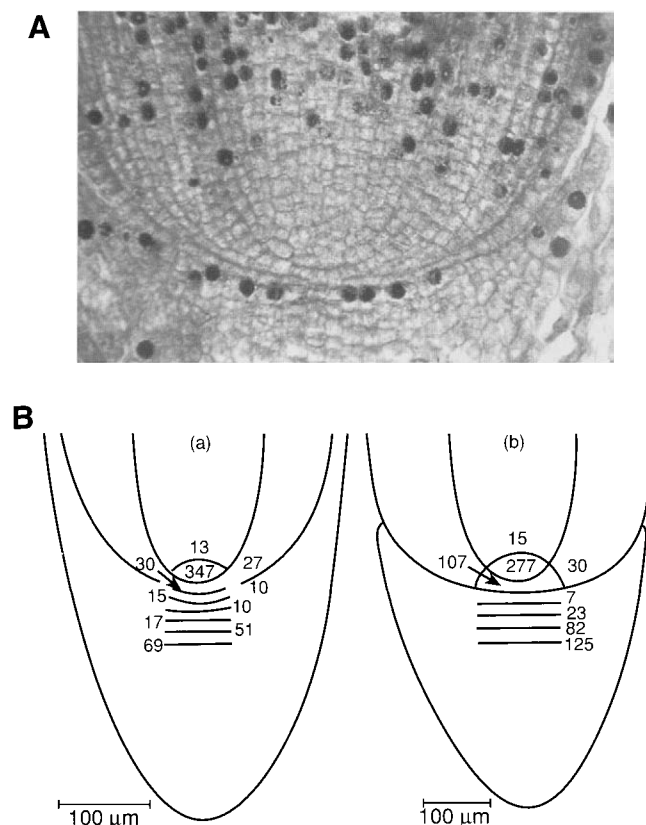


FIGURE 1-5 Cell divisions in the quiescent center and peripheral regions of root meristem. (A) Radioactive thymidine treatment of maize (*Zea mays*) roots. Cells in the peripheral parts of the root meristem show a greater incorporation of the radiolabel, which means that they are dividing more rapidly than those in the quiescent center. (B) Cell doubling time in hours in different parts of sunflower (*Helianthus annuus*) and maize roots. To calculate these times, root tips are sampled at intervals over a defined time period, sectioned, and examined for mitotic figures (colchicine is used to accumulate cells in metaphase). A large number of root tips in each sample provides statistically reliable counts [A, courtesy of Lew Feldman, U.C. Berkeley; B, with permission from Furuya (1984), © Annual Reviews].

both anchorage and absorption of water and minerals. In roots of woody dicots and gymnosperms, the vascular cambium is also initiated in the region, which is no longer elongating. The vascular cambium divides periclinally and produces secondary xylem and phloem.

3.2.2. Shoot Apex, Leaf Development, and Flowering Apex

The shoot and shoot apex are organized along entirely different lines from the root and root apex (Fig. 1-8A). The term **shoot** is used to denote stem and leaves together—the development of these two organs is inextricably linked and traceable to the shoot apex. The apex of the stem is covered over by a series of **leaf primordia**,

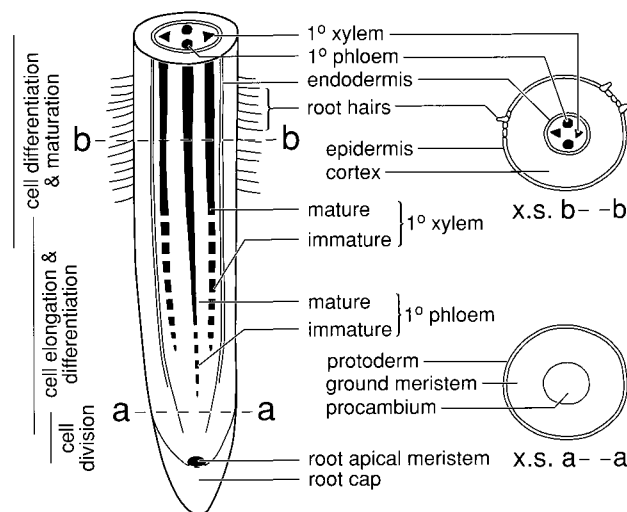


FIGURE 1-6 Diagrammatic illustration of growth and cell differentiation in a dicot root. The root tip is divided roughly into three zones on the basis of primary activities: cell division, cell elongation and differentiation, and cell differentiation and maturation. The zones partially overlap. Basipetally from the apical meristem, three primary tissues, protoderm (which gives rise to epidermis), ground meristem (which gives rise to cortex and endodermis), and procambium (which gives rise to xylem, phloem, and pericycle), are demarcated (see cross section at level a-a). Further basipetally, root elongation ceases and tissues and cell types mature. The cross section at level b-b shows all the primary tissues, epidermis (shown partly cellular to illustrate that root hairs are outgrowths of epidermal cells), cortex, endodermis, pericycle, primary (1°) xylem, and primary (1°) phloem.

with the youngest primordium being closest to the apex. To see an apex, these primordia must be surgically removed under a dissecting scope. A longitudinal section through the apex, with all but the youngest few leaf primordia removed, shows an apical meristem, which, depending on the species, may be dome shaped, conical, flat, or even concave. The term **shoot apex** is an inclusive term, whereas **shoot apical meristem** (SAM, or shoot meristem), by definition, is the part of the shoot apex above the youngest, discernible leaf primordium. In most flowering plants the shoot meristem shows an organization into a **tunica** and **corpus** [in gymnosperms and ferns, the apical organization is different, see Steeves and Sussex (1989) for details]. The tunica consists of one to several layers of cells at the periphery that divide predominantly in an **anticlinal plane**, i.e., at right angles to the surface; corpus cells, in contrast, divide in all planes (Fig. 1-8B).

Tunica and corpus refer to the organization of the apex. They do not give an indication of the frequency or site of cell divisions. As in the root apex, the central part of the shoot apical meristem, referred to as the **central zone**, is a region of low mitotic activity (Fig. 1-8C). The cells in this region, usually few in number, divide

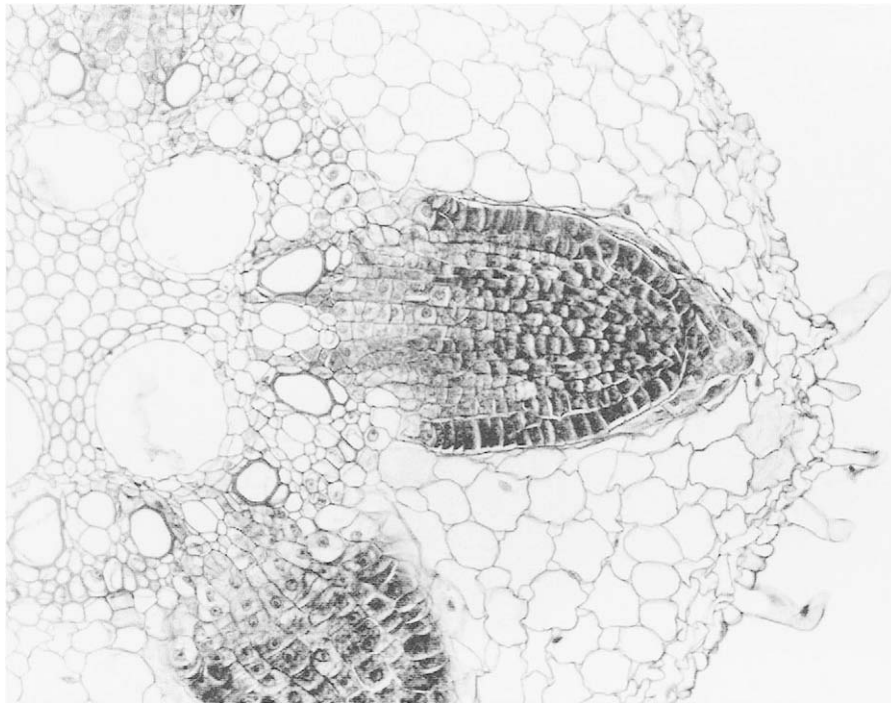


FIGURE 1-7 Origin of lateral root in maize (*Zea mays*). Lateral roots arise in the pericycle and grow through the cortex and epidermis of the parent root. Courtesy of Nancy Dengler, University of Toronto, Toronto.

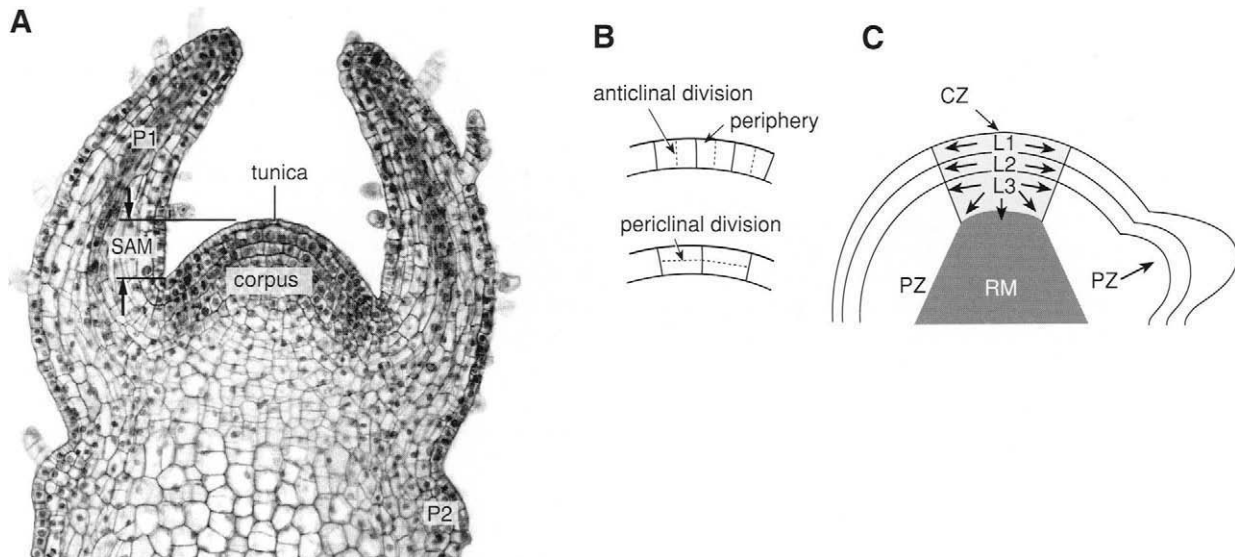


FIGURE 1-8 (A) Longitudinal section of the shoot apex of *Coleus*. The part above the youngest leaf primordium is considered the shoot apical meristem (SAM). It shows tunica layers surrounding a central mass of cells known as corpus. In *Coleus*, leaves are borne in an opposite decussate manner, which means that each succeeding pair of opposite leaves arises at right angles to the previous pair. The section passes through parts of two pairs of leaf primordia or young leaves (P1 and P2): P1, the youngest pair, is seen arching over the shoot meristem, whereas the P2 pair is seen as small protuberances (due to the plane of section) on the two sides of the stem. Trichomes (hairy outgrowths) are seen arising from the epidermis of P1. (B) Anticlinal and periclinal divisions. Cell divisions that are parallel to the periphery of root or stem are called periclinal divisions, whereas those that occur at right angles to the periphery are called anticlinal divisions. (C) Line sketch of a shoot apex showing the location of the central zone (CZ), peripheral zone (PZ), and the rib meristem (RM). Surface and subsurface layers are designated L1, L2, and L3. The CZ contributes cells to the surrounding ring of cells in the PZ as well as the RM below [A, courtesy of Nancy Dengler, University of Toronto, Toronto; C, from Liljegren and Yanofsky (1995) with permission from Elsevier Science].

slowly and give rise to cells below and on the sides to give rise to the **rib meristem** and the **peripheral zone**, respectively. Cell divisions occur mostly in localized areas in the peripheral zone, where lateral organs, such as leaves, are initiated, and in the rib meristem, which contributes to internodal elongation. The surface and subepidermal layers are designated L1, L2, and L3. L1 and L2 usually divide anticlinally, whereas L3 divides both anticlinally and periclinally. L1 and L2, and in most cases L3 as well, are continued into lateral organs and contribute to various tissues in these organs to varying extents.

3.2.2.1. Leaf Development

Leaves are highly plastic organs and show a great variety of form and size. Typical leaves are flat, dorso-ventral organs adapted for photosynthesis. Their arrangement on the shoot, known as phyllotaxy, is specific for each species. Knowing the phyllotaxy, one can predict with considerable accuracy where the future leaf, or leaves, would arise on the shoot apex.

Much of our recent information on leaf development in flowering plants has come from a study of cell lineages in genetic mosaics. Genetic mosaic, as the term implies, means an organ (or organism) that is composed of cells of more than one genotype. For studies in plant development, genetic mosaics are obtained in two ways: In periclinal chimeras, one or another cell in the outer surface (L1) or subsurface (L2 or L3) layers in the shoot meristem (usually CZ), shows a stable genetic change, the change spreads throughout the layer, and is perpetuated in subsequently produced lateral organs or axillary buds. The change itself may be chemically induced, e.g., polyploidy after colchicine treatment, or a natural mutation or it may be due to a graft from a different plant, but as long as it can be seen with naked eye or under the microscope, it serves its purpose. In clonal analysis, embryos in immature or mature seeds or seedlings may be mutagenized using ionizing radiation. As a result, some cells in the shoot apex or young leaf primordia may be mutagenized. These cells, when they divide subsequently, leave a record of their progeny (or clone). If the mutation is in some visible marker, e.g., loss of synthesis of chlorophyll or pigments such as anthocyanin, the clones can be easily followed during development.

These studies have revealed that the origin of most lateral organs can be traced to groups of cells, called **founder cells**, at the periphery of the shoot meristem. The group of cells spans all three layers of the meristem: L1, L2, and L3. For leaf development, the number of cells may range from about 5 to 10 cells per layer in *Arabidopsis* to somewhere between 50 to 100 cells per layer in tobacco, cotton, and maize.

The initiation of a leaf is signaled by the onset of a few periclinal divisions in the outer surface and sub-surface layers of the founder group, accompanied by a change in the orientation of growth. As a result, a bulge or leaf buttress is formed, which with continued growth gives rise to a leaf primordium. In dicots, the primordium often has a flat surface toward the shoot apex and a curved surface on the opposite side (Fig. 1-9). Subsequent growth of the primordium involves elongation by intercalary growth and growth at the margins to form a flat lamina, or blade. In grasses, leaf initiation follows a slightly different course. Mitotic activity in the founder cells spreads in the peripheral zone almost surrounding the shoot meristem before elongation growth starts. Thus, a hood-like primordium results and a sheathing leaf base is formed.

Subsequent leaf development is often analyzed in terms of three axes, which define the major parts of the leaf, its eventual shape, and the major tissue layers (see line drawing in Fig. 1-9B). The apical/basal axis defines the extent of the leaf blade (or lamina), which develops distally, and the petiole, leaf base, or leaf sheath, which develop proximally. The centrolateral axis defines the midrib and the lateral extent of the lamina. The dorsoventral (or the adaxial/upper and abaxial/lower) axis defines the extent of major tissues through the width of the leaf. All three layers participate to varying extents. L1 forms the epidermis, L2 gives rise to the inner subdermal layers, including upper and lower mesophyll, and L3 the innermost layers of mesophyll and vascular tissues (Fig. 1-10). Some inner tissues may be of mixed origin from L2 and L3. These definitions proceed with both cell divisions and differential cell expansion and lead to production of a fully mature leaf.

An important point to note here is that, in dicots and most monocots, although meristematic activity occurs in the leaf primordium and through most of leaf development, sooner or later it comes to a close—the meristematic potential of the primordium is used up during leaf development. Thus, while shoot (or root) apical meristems are indeterminate in nature, leaf meristems are determinate and leaf is a determinate organ. In grasses, an intercalary meristem at the base of the leaf continues to add new leaf tissue indefinitely, while the older, more distal parts differentiate and mature. Thus, it is that grass can be mowed.

3.2.2.2. Internodal Elongation

The site at which a leaf is initiated is designated a **node**; the intervening stem segment between two nodes is an **internode**. Cells in the ground tissue above each node, derived from the CZ (see Fig. 1-8C), divide

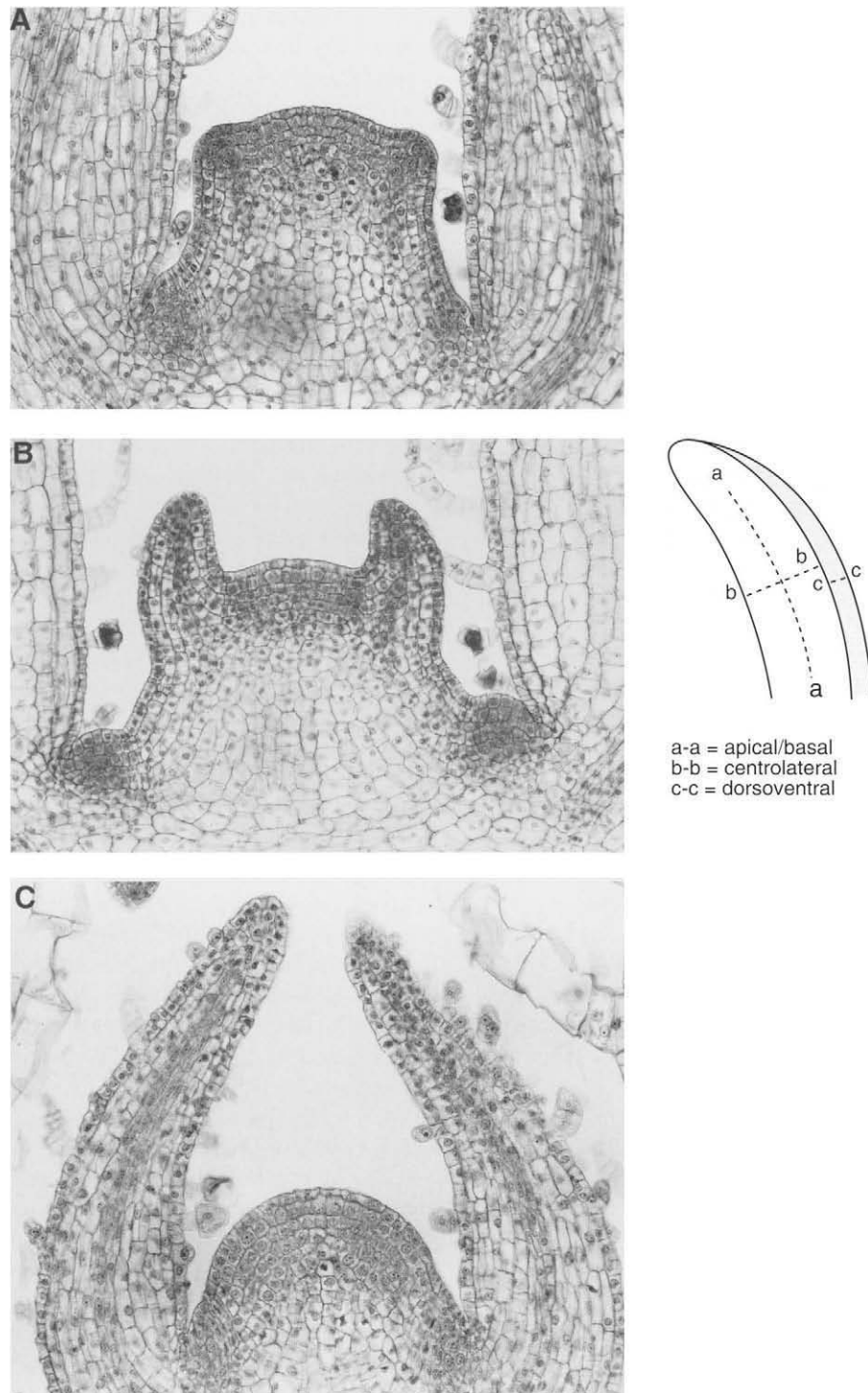


FIGURE 1-9 Leaf development in *Coleus*. Three stages in leaf development are shown. (A) A pair of very young primordia. (B) A slightly later stage. The accompanying line drawing shows the three axes of growth. (C) A pair of young expanding leaves with most of the major tissues and cell types defined. Courtesy of Nancy Dengler, University of Toronto, Toronto.

preeminently in a transverse plane, resulting in longitudinal files or ribs of cells, referred to as **rib meristem** (Fig. 1-11A). Cells derived from this activity elongate,

resulting in growth in length of the internode. In most plants, such extension growth continues over several internodes below the apical meristem. As a result

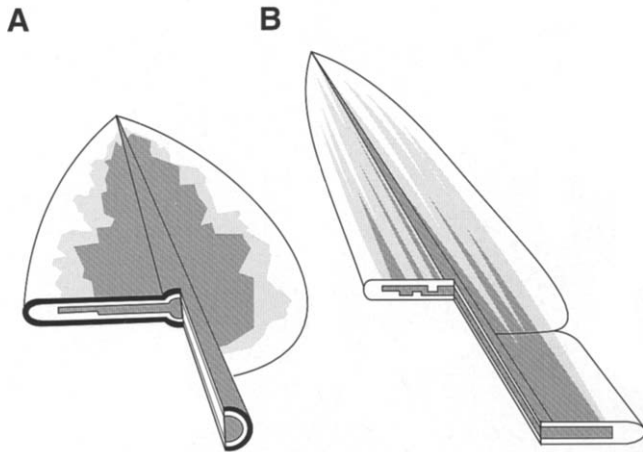


FIGURE 1-10 Distribution of tissues derived from L1, L2, and L3 layers of the shoot apical meristem in a tobacco leaf and a maize leaf. (A) A leaf from a green (L1)–white (L2)–green (L3) periclinal tobacco chimera. In tobacco, the L1 lineage is confined to the epidermis. Most of the tissue at the margin of the leaf is derived from the L2 lineage because of periclinal divisions in this layer early in the expansion of the lamina. (B) A leaf from a white (L1)–green (L2)–green (L3) periclinal maize chimera. In maize, the L1 layer produces epidermis, plus all of the tissue at the margin of the leaf, whereas the L3 layer produces little, if any, of the tissues in the lamina. From Poethig (1997).

of this elongation, the shoot apex and the young leaf primordia are constantly carried upward. Sooner or later, however, the older internodes cease elongation. Thus, the primary extension or elongation growth of the stem occurs only in a few internodes below the apical meristem; the rest of the stem does not elongate. There are some plants, such as celery, lettuce, and cabbage, where the leaves and shoot apex stay close to the ground, because while the leaves are initiated and formed, internodal elongation below the apical meristem is suppressed. These plants are called **rosette** plants. Likewise, in many dwarf varieties of wheat, maize, and pea, which are genetic mutants, the internodal elongation is severely curtailed.

Differentiation of primary tissues, protoderm, ground tissue, and procambial strands accompanies or follows the extension growth of the stem. Procambial strands, which later differentiate as primary xylem and primary phloem tissues (Fig. 1-11B), extend into the newly developing leaf primordia and, within the stem, form an interconnected system of vascular strands.

3.2.2.3. Lateral Bud Initiation

Lateral buds are usually initiated at the flanks of the shoot meristem by localized cell divisions in the axils of leaves (the angle between the leaf and the stem). The lateral bud acquires its own shoot apex and leaf primordia and is a miniature shoot. In herbaceous plants and shrubs, which show abundant branching, it may

grow into a branch with expanded leaves soon after its initiation (e.g., two newly initiated buds and two young branches are seen in Fig. 1-11). In plants with a strong dominant main shoot(s), lateral buds close to the main shoot apex stay dormant for a long time; however, if the dominant shoot is cut or injured, they grow out and one of them forms a new dominant shoot. In perennial plants, buds produced near the end of the growth season first produce a few scale leaves followed by a few normal foliage leaves, which stay unexpanded, and with little internodal extension. Scale leaves ensheath and protect the apical meristem and the young leaves until growth resumes (Fig. 1-12).

In many plants, the close association between the site of lateral bud initiation and a leaf described earlier is not evident. Also, in many cases, buds arise at nodes further down in mature parts of stem, probably from progenitor cells derived from shoot meristem that were left in a quiescent state to take up cell division and bud formation activity later.

3.2.2.4. Floral Apex

The transition from vegetative to flowering shoot apex is accompanied by dramatic changes not only in the structure of the apex, but also in patterns of cell division and growth and the nature of derivatives (Fig. 1-13). The tunica-corpora organization of the vegetative shoot meristem is lost and cell divisions spread throughout the apical meristem. Instead of leaves and buds being formed, floral parts—sepals, petals, stamens, and gynoecium (or pistil)—are initiated and there is little elongation growth between initiation of these parts. As a result, the floral parts are borne on a condensed axis, and the flower is a condensed shoot. The shoot (or floral) apex is used up in the formation of the floral parts and its meristematic potential is terminated. Floral parts, like leaves, are determinate organs, and the flower is a determinate shoot.

There is an infinite variation in the structure of flowers: their size, number, and presence or absence of individual floral organs, symmetry, fusion of floral organs with each other and with members of adjacent whorls (organs of a different type), and position of gynoecium (specifically ovary) with respect to other floral organs. These variations are ultimately traceable to the patterns of cell division and growth in the floral apex. Flowers may also be borne in clusters (**inflorescences**). In some inflorescences, such as racemes (e.g., fox glove, *Digitalis purpurea*) and panicles (e.g., oat, *Avena sativa*), the original apex may continue its activity for considerable periods while producing laterals, which terminate in flowers. In others, such as a head (e.g., sunflower, *Helianthus annuus*), the inflorescence axis is condensed and many flowers are borne spirally on the condensed axis.

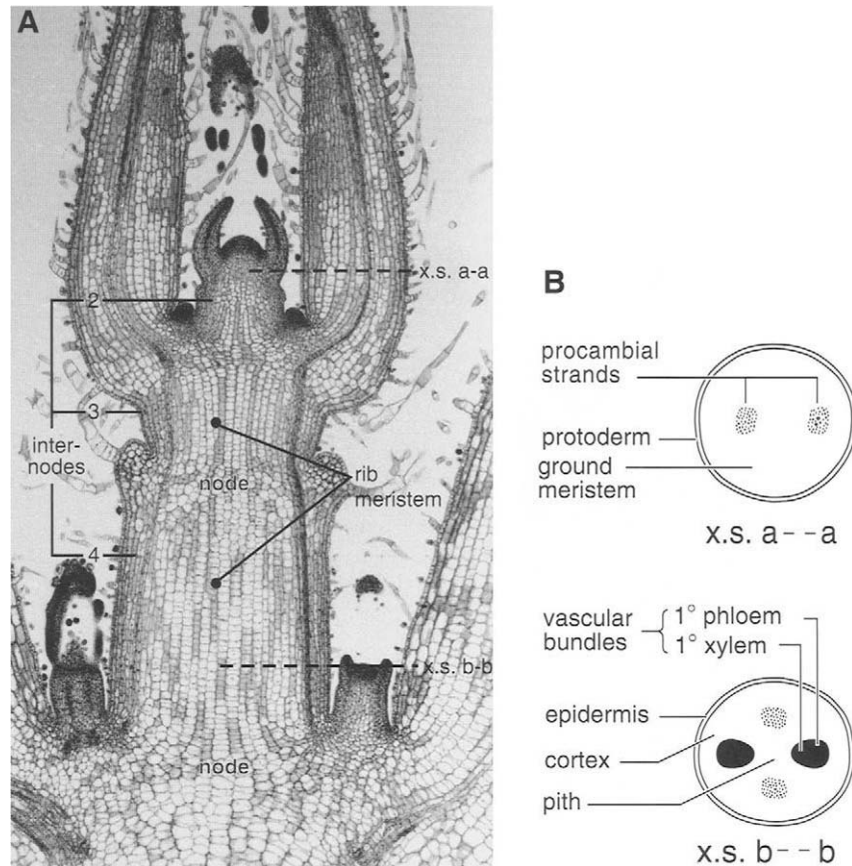


FIGURE 1-11 Extension growth and differentiation of primary tissues in a dicot stem. (A) Rib meristem activity in a *Coleus* stem. Five leaves, including the youngest leaf primordium, and four internodes, appear in this section. Note the longitudinal files of transversely dividing cells, the result of rib meristem activity, in internodes 3 and 4. In older internodes further down, this activity ceases and derivative cells stop elongating with the result that the internodes also cease elongation. Courtesy of Nancy Dengler, University of Toronto, Toronto. (B) Simplified drawings of a dicot stem in cross sections at two levels illustrating the differentiation of primary tissues (x.s. at level a-a) and primary xylem and phloem in procambial strands (x.s. at level b-b).

3.2.3. Secondary Growth and Vascular Cambium

In gymnosperms and woody dicots, a vascular cambium makes its appearance in that region of root or stem that has ceased elongating and produces secondary xylem and phloem. The addition of secondary vascular tissues, especially xylem, adds to the girth of these organs and provides the needed structural support to trees. Small amounts of secondary growth may also occur in some species in petioles and midveins of leaves and in axes that bear flowers, but because these organs have only a limited life span, it is never extensive. Many herbaceous dicots also develop a cambium, but it may

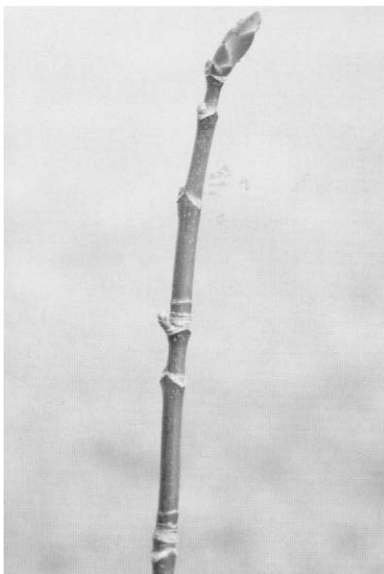


FIGURE 1-12 A terminal and several lateral buds in maple (*Acer saccharum*) at the time of bud break in spring. These buds have a shoot apex and a few scale leaves followed by a few vegetative leaves. There is little internodal elongation. Scale leaves, seen well in the terminal bud, enclose and protect the lateral shoot until it is induced to grow.

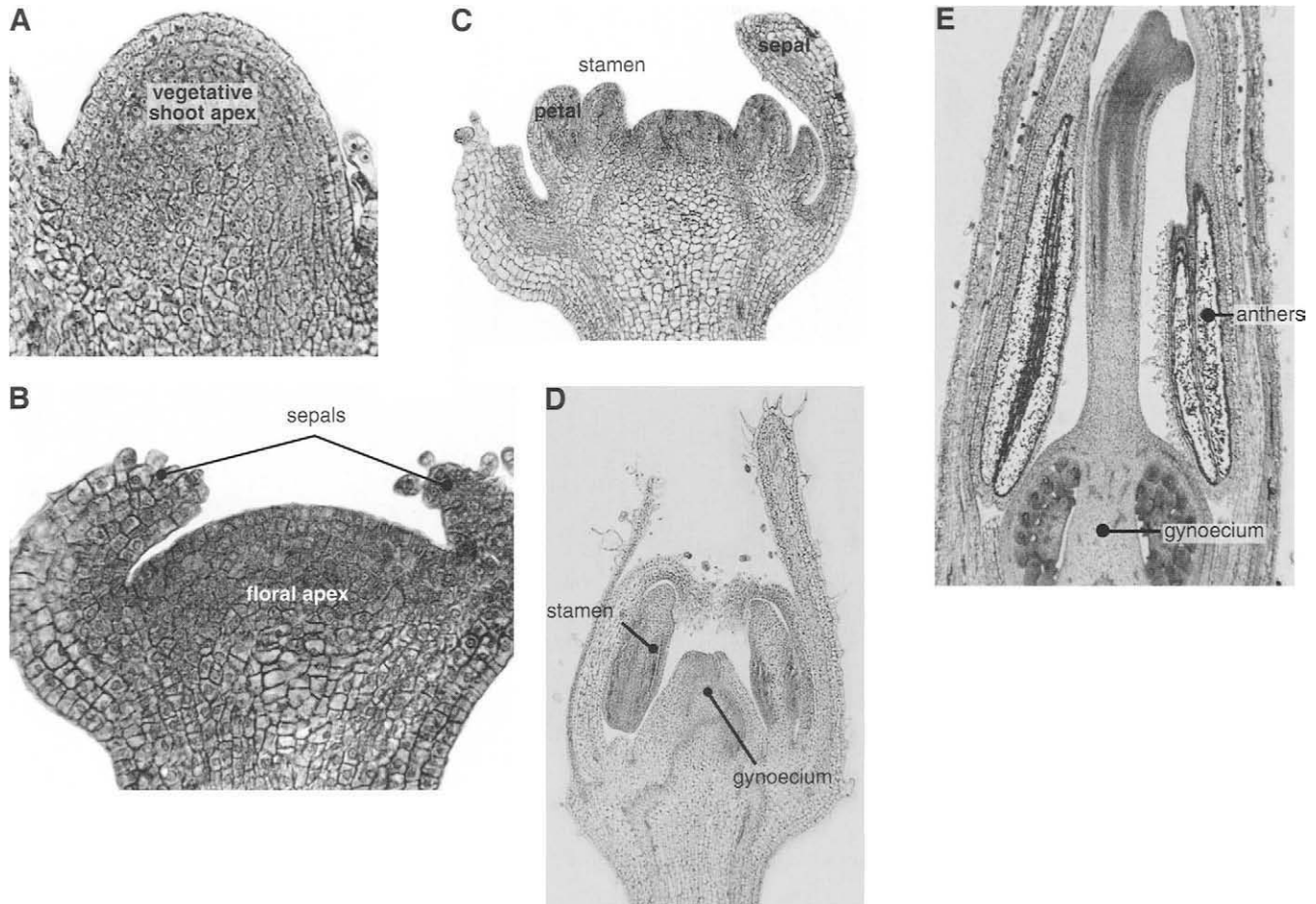


FIGURE 1-13 Longitudinal sections showing the transition from a vegetative to a floral meristem and the development of floral organs in tomato (*Lycopersicon esculentum*). (A) Vegetative shoot meristem. (B) Transition to flowering meristem; sepals have originated. (C) Origin of petals, stamens, and gynoecium. Sepals are in the outermost whorl. (D) Later stage in the development of stamens and gynoecium. (E) Further development and maturation of gynoecium. Anthers, the pollen-bearing structures in stamens, are fully mature. Note that the floral parts develop in a certain order. In tomato, sepals are the first to be formed and mature gynoecium is the last. Photographs are at different magnifications. Courtesy of Vipen Sawhney, University of Saskatchewan, Saskatoon.

not form a complete ring and its activity may be restricted to the vascular bundles.

The vascular cambium is a layer of meristematic cells (or initials) that arises between primary xylem and phloem. Although it is a single layer of cells, in actual practice it is difficult to distinguish that layer from its immediate derivatives on either side. Hence, the term cambial zone is used (Fig. 1-14A). With few exceptions, the cambium consists of two types of initials; the fusiform and ray initials (Fig. 1-14B–D). Fusiform initials are elongated cells that divide periclinally and give rise to axially elongated cells in the xylem and phloem, i.e., is, tracheary cells, sieve elements, fibres, and parenchyma cells or vertical files of parenchyma cells, called parenchyma strands. Ray initials are more or less isodiametric and occur in clusters that appear spindle shaped in tangential sections. Ray initials give rise

to xylem and phloem rays, which extend radially into the xylem and phloem and provide for the radial transport of water, minerals, and photoassimilate.

The vascular cambium originates in roots and stems in slightly different locations (for origin in stems, see Fig. 1-1), but eventually in woody plants it forms a complete ring—it extends up and down the stem or root like a cylindrical sheath. How this sheath of cells with two distinct types of initials and a specific spatial arrangement comes to originate in procambial strands has not been studied closely and the details of transition are unknown.

Procambial strands are composed of narrow elongated cells. In dicots and gymnosperms, some of these cells escape differentiation as primary xylem or phloem cells and are left in a potentially meristematic state. Most likely, some of these cells become committed

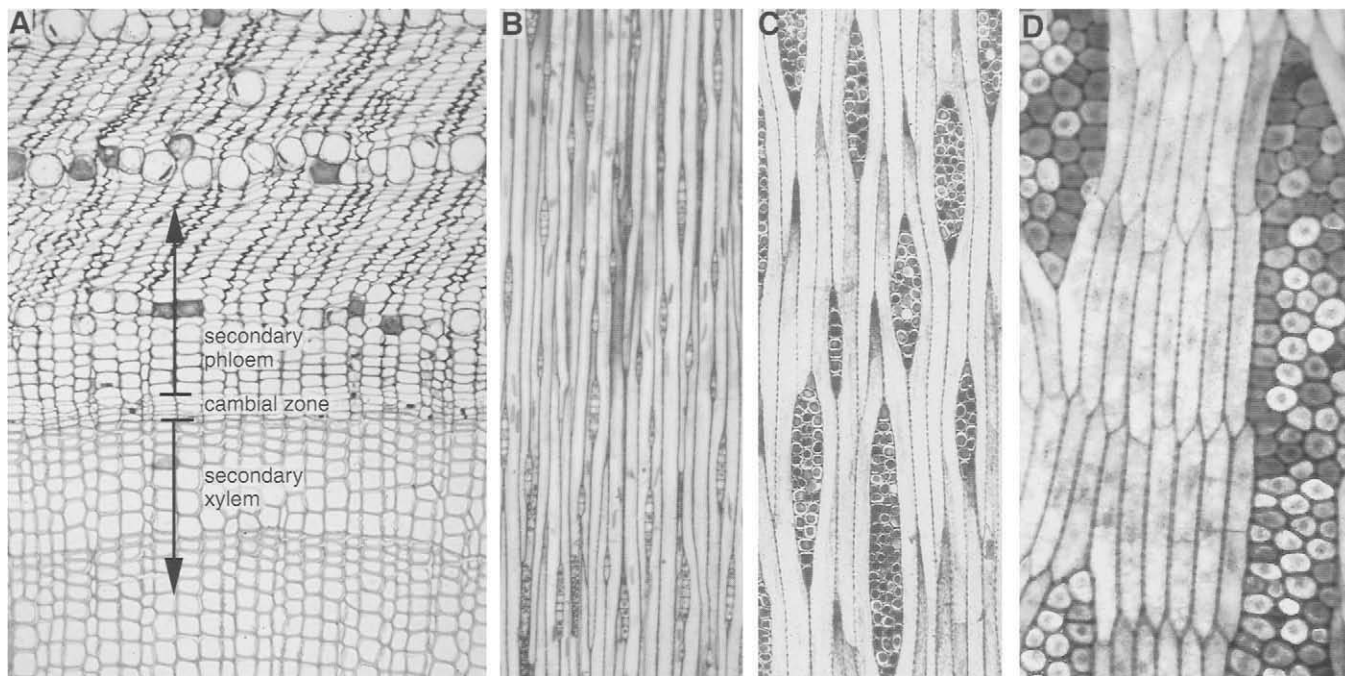


FIGURE 1-14 (A) Cross section of a pine (*Pinus* sp.) stem showing the location of the vascular cambium, secondary xylem, and secondary phloem. Tangential longitudinal sections through cambia of three woody trees, pine (B), birch (*Betula* sp.) (C), and black locust (*Robinia pseudo-acacia*) (D), showing the arrangement and orientation of the fusiform and ray initials. Note that in pine and birch the fusiform initials have ends that overlap with each other, whereas in black locust they are in tiers one upon another. Cambia with the former type of arrangement of fusiform initials are referred to as nonstoried cambia, whereas those with latter type of arrangement are referred to as storied cambia. Also note the differences in the width and the height of rays in the three species. Reproduced with permission from Arnoldia (1973).

as fusiform initials, which, likewise, are elongated cells, whereas others give rise to ray initials after divisions. The actual process is probably more complicated and occurs over some time, but eventually results in the conferment of a new polarity, which is unique to cambium. Cambial cells divide in a strict periclinal plane and give rise to derivatives whose destinies are predetermined as xylem or phloem cells.

Cambium is not, however, a static cell layer placidly cutting out derivatives on each side, which differentiate as xylem and phloem cells; rather it is a seat of constant and dynamic change in interrelationships among fusiform and ray initials. In addition to dividing periclinally, cambial initials also divide periodically in an anticlinal plane (at right angles to the periphery of the stem or root) to add to their numbers and thus cope with the increasing diameter of the wood cylinder, a result of their own activity. In cambia that have been studied in detail, fusiform initials divide anticlinally with much greater frequency than required—far more cells are produced than needed. Excess cells are converted to ray initials by further divisions or they cease dividing and are lost from

the cambial ring by differentiating as xylem or phloem cells. As a result, interrelationships among cambial initials are constantly changing and confer upon the cambium an added measure of plasticity. Such plasticity is useful in accommodating pathogens, such as mistletoe, which draw nutrients from host xylem and/or phloem, or in producing more wood on one side to cope with gravity or other environmental stresses, such as snow drifts and leaning boulders.

4. PLANT DEVELOPMENT INVOLVES COMMITMENTS

“Commitment” or “determination” is a general term that includes setting up of polarities and pattern formation. Inherent in the concept of polarity is the presence of poles, typically two, with an axis running between them, thus apical–basal polarity, or in and out (radial) polarity. In contrast, patterning of organs, such as leaf or flower, may show little polarity, but nonetheless are examples of commitment.

The establishment of polarities and patterns during development (e.g., establishment of the root and shoot poles, the distinctive organizations of the root and shoot apices and their respective patterns of growth, the establishment of the vascular cambium and its precise patterns of division and nature of its derivatives) are an intriguing developmental phenomena, which are still only partly understood.

Some of these topics are covered in Chapters 3 and 4. Here it is important to note that commitments occur at different times in plant development. Some are established early in development, e.g., the first division of the zygote; others are established later, e.g., root and shoot poles in the heart-shaped embryo; and still others are established even later, e.g., cambial activity after elongation growth has ceased. Thus, plant development is hierarchical in nature and involves a series of progressive commitments. The second thing to note is that once established, these commitments stay throughout the life of the plant. They confer on the plant a sense of up and down and in and out, what may be considered an apical/basal polarity and a radial polarity. These polarities are essential for an orderly growth of the plant. They are maintained throughout the life of the plant unless perturbations occur in its environment.

There are numerous examples to show commitments and their stability. Two are given here.

4.1. Determined State of Meristems

The apical root and shoot meristems, and lateral meristems, such as vascular cambium, are unique tissues in that they retain their determined state while continuing to divide and produce derivatives that go on to differentiate as different cell types. Some authors have distinguished between “proliferative” and “formative” cell divisions. The former allow meristematic cells to perpetuate themselves while retaining their determined state, whereas the latter allow derivative cells to pass to the next stage of determination or differentiation.

A good example of meristems retaining their determined state is provided by root tips in culture. Excised root apices kept in culture continue to grow almost indefinitely, they can be subcultured, but all they produce is root tissue. One such culture started in Jackson Laboratories, Bar Harbour, Maine, in 1927, was still going in 1968 (Fig. 1-15).

Excised shoot apices in culture also produce stem tissues and leaves and, at least in early stages of culture, no roots. Later, because of the production of endogenous hormones, they produce roots as well and form whole plants. These experiments suggest that root and shoot apices, once determined, continue to produce root and shoot tissues, respectively, unless some

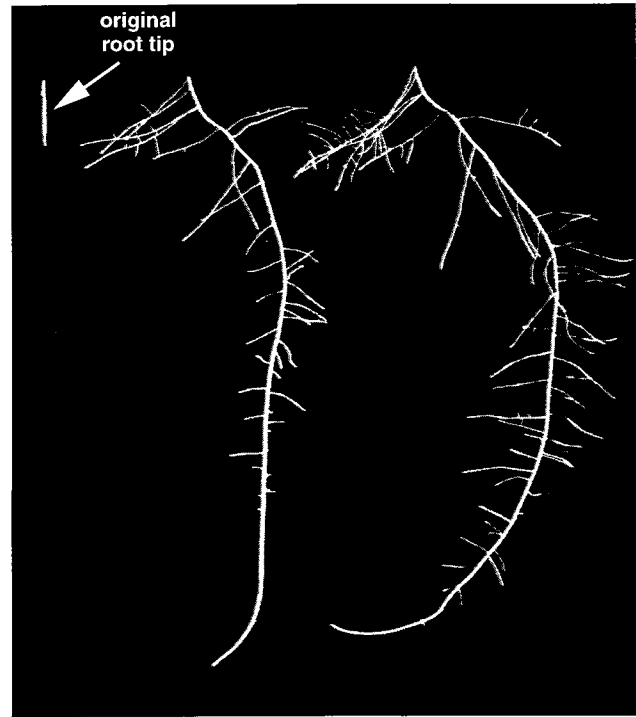


FIGURE 1-15 Root tips in culture. A typical clone, age 355 days, which had been subcultured 23 times, was used as the source for the root tip shown at the upper left; middle figure, 12 days later, and figure on the right, 4 more days later. Slide courtesy of the late Phillip White.

additional perturbations occur. Appropriate hormonal treatments can provide a further stimulus for roots to produce shoots and for shoots to produce roots.

Similar culture experiments with vascular cambium have been tried, but without success. However, a slightly different type of experiment has been done with vascular cambium. If a square block of tissue, including the cambium, is lifted off a tree trunk (it is possible to do this in the first flush of spring growth when the bark slips—the break occurs in the young xylem cells, not cambium), rotated by 90°, replaced, and some judicious pressure applied on the block, the wound heals in time, and the lifted block continues to produce xylem and phloem cells on the two sides, but the new cells that are produced are elongated horizontally in line with the orientation of the fusiform initials and not vertically as the neighboring fusiform initials and their xylem and phloem derivatives (Fig. 1-16). Some pressure is needed for this orderly continuation of the cambial activity because, in its absence, fusiform initials divide up into numerous small cells and form an unorganized tissue mass, known as the **callus**. The callus may eventually show organization and differentiation, but that is another story.

If a cambial explant is lifted off and put on solid culture medium, it again forms a callus, which may

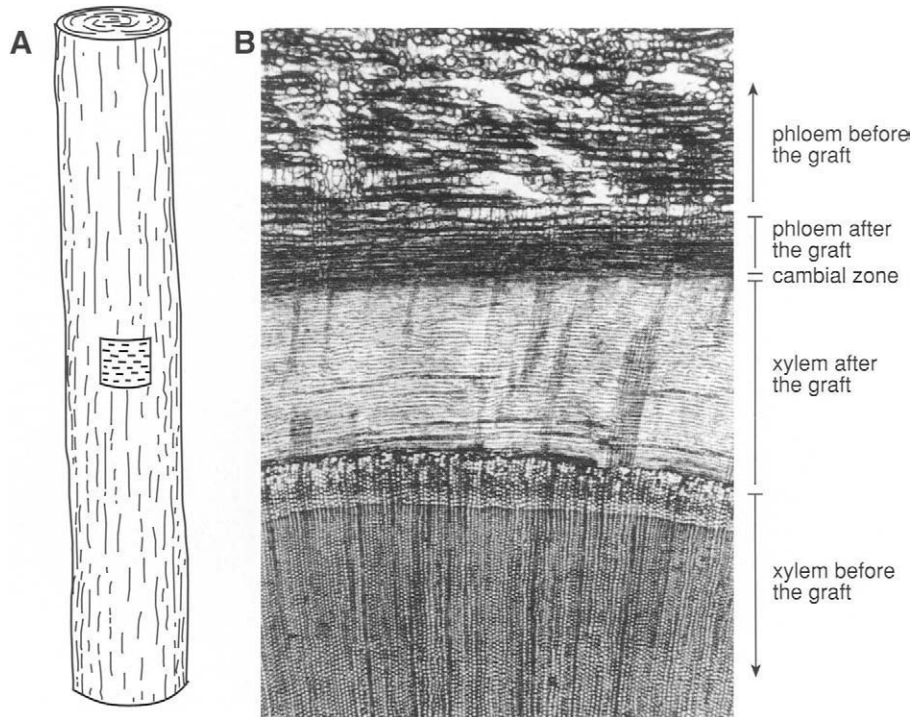


FIGURE 1-16 Determination in vascular cambium. A square block of bark tissue (all tissues external to young differentiating secondary xylem) is lifted off, replaced after being turned by 90°, and some pressure applied (A). The graft takes hold and the new secondary xylem and phloem are produced in line with the changed orientation of the cambial initials at right angles to the previously produced secondary xylem and phloem (B). From Thair and Steeves (1976).

continue to divide, albeit at a slow pace, but lacking the precise orientation of cell divisions as well as the earmarked destiny of the derivative cells, two properties that characterize the cambium.

4.2. Polarity in Shoot and Root Cuttings

A common horticultural practice to propagate plants, especially dicots, is to take stem cuttings and get them to root by putting them in soil or moist air. Adventitious roots are produced at the morphologically basal end of the stem, not the apical end (Fig. 1-17). If the stem segment is inverted, the roots are still produced by the morphologically lower end. Conversely, the shoots are produced at the morphologically upper end. Even though the two cut ends look the same, they are physiologically distinct. Experiments demonstrating this physiological polarity were performed by Julius Sachs more than 100 years ago.

Similar experiments showing physiological polarity can be conducted with cut roots as well, e.g., dandelion roots, where the shoots are produced from the proximal end, that is, the end closer to the root-shoot junction, whereas new roots are produced near the

distal end, that is, the end farther away from the root-shoot junction.

5. EXTERNAL OR INTERNAL PERTURBATIONS MAY CAUSE A REVERSAL OF ESTABLISHED COMMITMENTS

The established commitments are not immutable. Plants respond to external or internal perturbations, such as a change in environment, pathogen attack, wounding, hormonal imbalance, in various ways, and in some cases by a reversal of their established commitments. The extent of reversal, whether partial, i.e., going back a few steps, or complete, going back to the zygotic stage, seems to be a function of the extent of perturbation. Two terms, **dedifferentiation** and **redifferentiation**, are used to denote a reversal of established patterns and differentiation along new lines. Cell divisions play important roles in many of these reversions.

The following examples show a reversal of commitments.

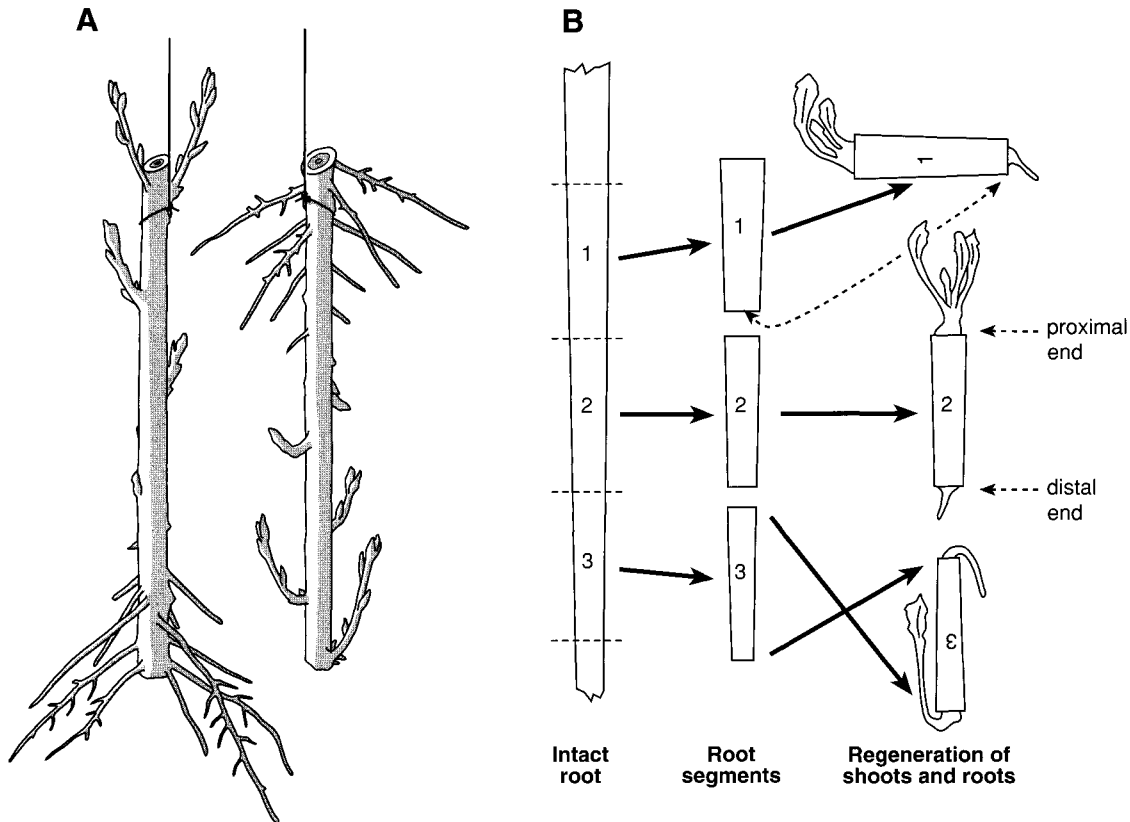


FIGURE 1-17 Polarity in shoots and roots as shown by induction of rooting in cut stems and of shoot buds in cut roots. (A) Stem cuttings of a willow (*Salix*) suspended in moist air in normal and inverted orientations. Roots grow out at the morphologically lower end and shoot buds at the morphologically upper end, regardless of orientation. (B) Roots from plants such as dandelion (*Taraxacum*) or chicory (*Cichorium*) can be segmented and placed in varying orientations. Shoot buds develop at the end originally furthest from the root tip (proximal), and roots develop at the end toward the root tip (distal), regardless of orientation. From Wareing and Phillips (1981) and Warmke and Warmke (1950).

5.1. Production of Whole Plants from Leaf Tips or Margins

Many plants, such as the succulents, *Bryophyllum*, and *Crassula*, Mexican Hat plant, *Begonia*, *Saint-paulia*, reproduce vegetatively by producing small plantlets with leaves, stem, and roots along leaf margins (Fig. 1-18). In time, these plantlets fall off the leaf and establish themselves as new plants. In *Bryophyllum*, groups of cells at leaf margins are left undifferentiated to produce these plantlets; in others, seemingly mature cells at leaf margins or leaf surface resume meristematic activity and the products differentiate into root, stem, and leaf primordia. In the orchid, *Malaxis padulosa*, clusters of small globular embryos are produced at the tip of a mature leaf, which later fall off and grow as new plants. In this case, also, mature cells at the leaf tip resume meristematic activity and form embryo-like structures.

5.2. Production of Adventitious Roots and Shoot Buds

We saw earlier production of roots from stem cuttings and, conversely, production of shoot buds on root cuttings. These things occur naturally as well. Many climbing plants, such as ivy, produce adventitious roots from the stem tissue. These roots produce a sticky material, which is useful in anchoring the plant to the support (wall or a tree trunk). Likewise, many plants, such as poplar, black locust, and redwood, produce shoot buds on their roots—they are called **root sprouters**; in time, shoot buds grow out as new plants far away from the main plant.

In the production of roots from stems, cell divisions occur in parenchyma cells in the vascular region (stem tissues in angiosperms and gymnosperms lack a pericycle) and new rootlets are initiated that break through the stem cortex and dermal tissue (Fig. 1-19). The formation of shoot buds on roots seems to occur in the

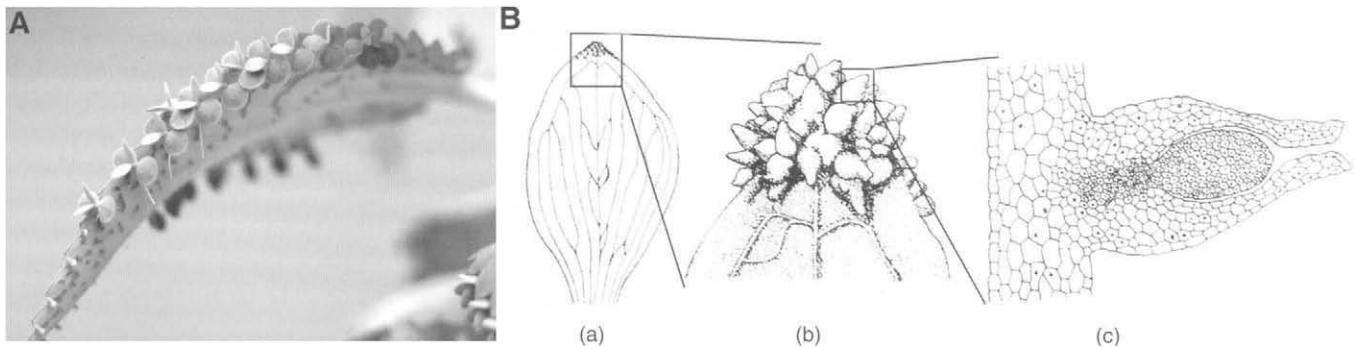


FIGURE 1-18 Production of foliar embryos in the Mexican Hat plant (A) and in *Malaxis padulosa* (B). Demarcated areas in B (a and b) are shown at higher magnifications (b and c, respectively) From Steeves and Sussex (1989).

same manner as that of lateral roots—cell divisions occur in pericycle or vascular parenchyma to produce a group of cells that later gets organized as a shoot bud. In both cases, cells with established destiny undergo partial dedifferentiation followed by redifferentiation along new lines.

5.3. Cells in Culture Can Dedifferentiate Completely

Under certain conditions, complete dedifferentiation can occur. If a cambial explant is put in a liquid culture and shaken gently, fusiform and ray initials lose their precise spatial arrangements and planes of division altogether, they divide in all planes and become similar to “undifferentiated” parenchyma cells (Fig. 1-20). They lose their differentiated or determined state and become ordinary parenchyma cells.

This leads us to the well-known phenomenon of regeneration of whole plants from single cells, other

than zygote, or **somatic embryogenesis**. In the early years of the 20th century, Haberlandt had speculated on the potential totipotency of plant cells. Almost 50 years later, and after numerous attempts by different scientists, Steward *et al.* (1957) showed that if explants from carrot roots were put in liquid culture supplied with coconut milk and the flasks were rotated gently, single cells were dislodged from their neighbors and showed asymmetric divisions typical of embryo development. These young “embryoids” could be removed from the flask and planted on solid agar to give in time mature carrot plants, which flowered and set seed normally (Fig. 1-21).

This landmark work has been repeated in hundreds of laboratories around the world, with different plants and different sources for the initial inoculum. This

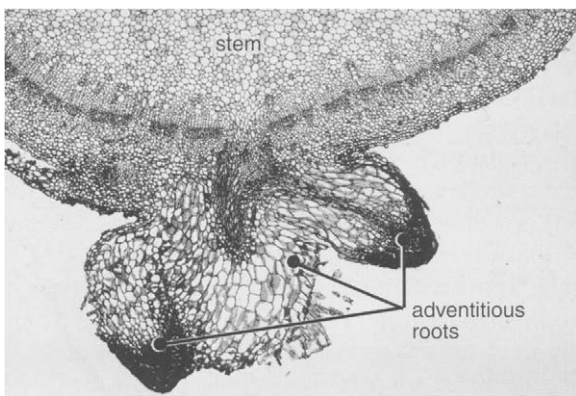


FIGURE 1-19 Production of adventitious roots in stems of climbing ivy (*Hedera helix*). A transverse section of the stem is shown with parts of three adventitious roots.

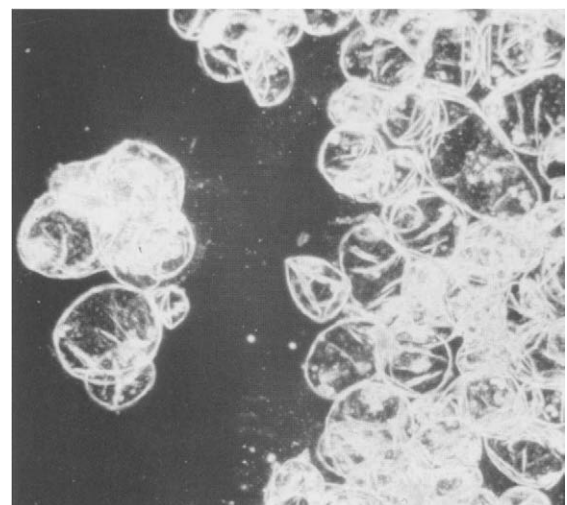


FIGURE 1-20 A cambial explant from sycamore (*Acer pseudoplatanus*) was placed in liquid culture medium and shaken gently. Cambial cells divide in all planes and lose their determined state. Courtesy of Peter Albersheim.

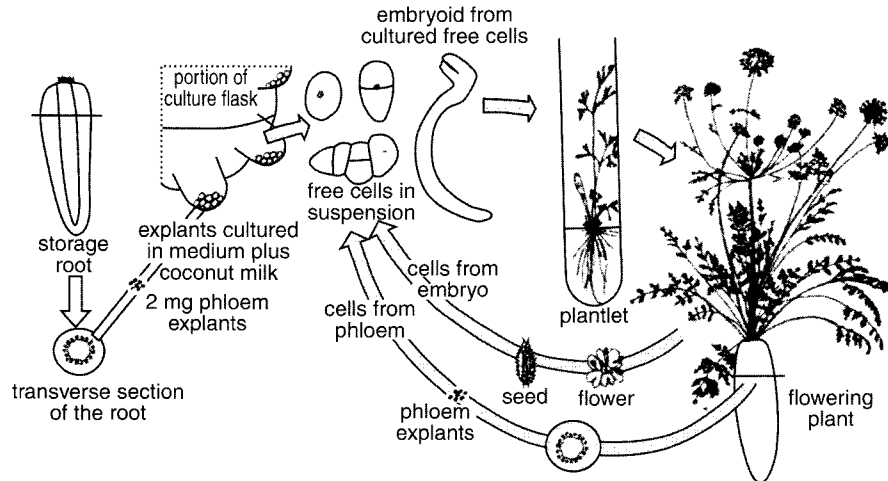


FIGURE 1-21 Production of whole plants from single cells in carrot. From Steward (1968).

capacity of single cells, other than zygote, to produce whole plants, the so-called **totipotency** of plant cells, is covered in Chapter 3. It is a standard tool utilized in the modern-day genetic engineering of plants.

6. CHAPTER SUMMARY

This chapter reviewed the basic aspects of plant embryogenesis and development of the adult body to serve as a backdrop for other chapters in Section I of this book. Plant development is characterized by an open form of growth, which means that it occurs continuously throughout the life of the plant because of the activities of the root and shoot apical meristems and vascular cambium. New, but same, body parts and tissue types are produced iteratively with a possibility for change as environmental factors change with time. Plants also show open differentiation. Body parts, tissues, and cells do become committed, they do differentiate and specialize for a function, but they retain the ability under certain circumstances to turn back the clock, partially or all the way back to the zygotic state, and enter a new developmental program. Chapter 4 considers the phenomena of determination, differentiation, and dedifferentiation. Here it is important to emphasize that both open growth and open differentiation confer upon plants a unique ability to alter their growth patterns depending on changes in their environment. This plasticity of growth is generally not available to most higher animals. These are strategies that plants, being rooted, have evolved to survive on earth.

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2

Cell Wall, Cell Division, and Cell Growth

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SECTION I. CELL WALLS

1. CELL WALLS SERVE MANY IMPORTANT FUNCTIONS

A distinguishing feature of plant cells is the presence of a cell wall outside the plasmalemma. Although considered as nonliving, the cell wall is a very important entity and has a role in several important functions. It provides strength and rigidity to the cell and, hence, the plant. At the same time, it is so well put together in terms of its constituents and architecture that it allows growth and expansion without sacrificing strength. Among other functions, it provides intercellular adhesion, pH and ionic balance in extracellular space, porosity to macromolecules, and intra- and interspecific recognition; in epidermal cells, it also provides protection against desiccation and pathogens.

Commensurate with their multiple functions, cell walls are very complex structures and represent one of Nature's marvels of structural engineering. This chapter considers mainly the walls of parenchyma cells. These cells are the most common in plants, they are the ones involved in cell division and cell growth, and all other cell types, at least in the vegetative body, arise from modifications in their walls. The other cell types include lignified cells, such as tracheary elements, fibers, and sclereids; cork cells that have suberin; and collenchyma cells that have intercellular wall thickenings. These special cell types are considered only briefly. This chapter also shows how the presence of the cell wall affects cell division and growth.

2. WALL LAYERS

Cell walls of parenchyma cells can usually be separated into two distinct regions. An **intercellular wall substance** or **middle lamella** (ML), which cements two cells together, and the **primary wall**. The common wall between two cells thus appears as a three-ply structure, bounded on each side by the plasma membrane (PM or plasmalemma) of each cell (Fig. 2-1A). In nonmeristematic tissues, parenchyma cells often round off, or separate from each other. They

separate along the middle lamella, creating **intercellular spaces** (ICS). These ICS form an interconnected system through much of the plant and serve an important role in aeration and gas exchange (Fig. 2-1B).

Primary walls are typical of cells in growing regions, they may be thin or thick, and may even show distinct layers, as in epidermal cells. All cells in the plant body have primary walls. In some cells/cell types, after cell expansion has ceased, a **secondary wall** is deposited between the primary wall and the PM. In xylem tracheids and fibers, the secondary wall may be three layered, a thin secondary₁ (S₁), a relatively wide S₂, and again a thinner S₃. In sclereids, or stone cells, in contrast, a secondary wall with many layers of nearly equal width may be deposited. During the differentiation of tracheary or stone cells, the secondary as well as the primary wall and ML become encrusted with lignin, a phenolic substance. Lignification renders the cell walls rigid and unable to grow. Not all cells that deposit a secondary wall become lignified. In sieve elements of many plants, a thick polylamellate secondary wall is deposited during differentiation, but the wall does not become lignified. In cork cells in the periderm, suberin, a complex of esterified hydroxy fatty acids and phenolic substances, is deposited in the walls and renders them impervious to water and air.

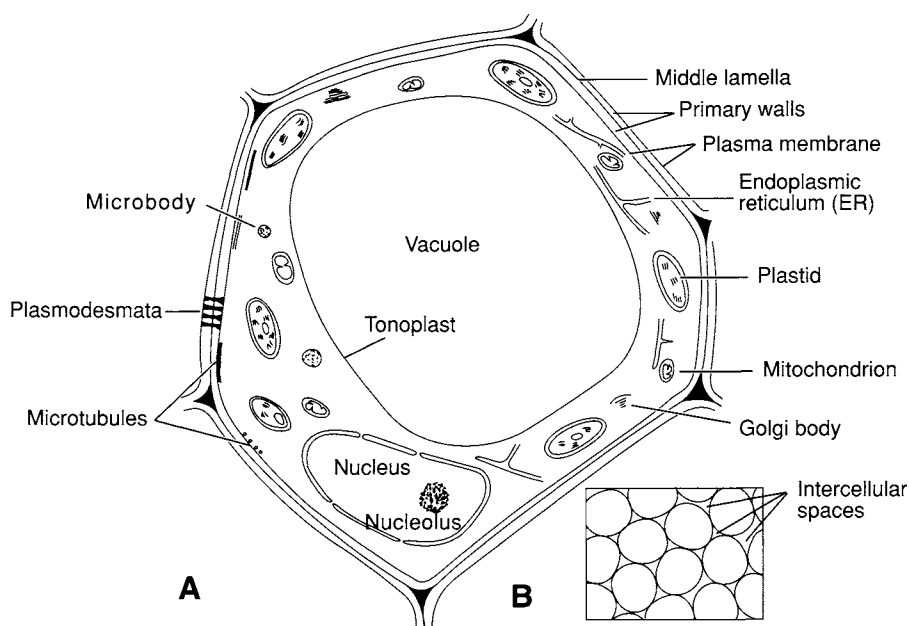


FIGURE 2-1 (A) Diagrammatic representation of a parenchyma cell showing the major cell organelles and membranes, and middle lamella and primary wall. (B) Separation of cells along the middle lamella creates intercellular spaces (ICS), which are important for aeration.

3. WALLS OF PARENCHYMA CELLS

3.1. Chemical Constituents in Walls of Parenchyma Cells

Walls of parenchyma cells are composed of several different types of materials, polysaccharides, proteins, phenolic compounds, and, in some special cell types, lipids.

3.1.1. Polysaccharides

Polysaccharides are polymers of five or six carbon sugars, such as arabinose, glucose, mannose, and galactose; seven such sugars provide the building blocks in most cell walls (Fig. 2-2). Some polymers, called homopolymers, have only one type of sugar; they are named after the constituent sugar, e.g., arabinan, xylan, and glucan. Others, called heteropolymers, have two or more sugars. Heteropolymers are known

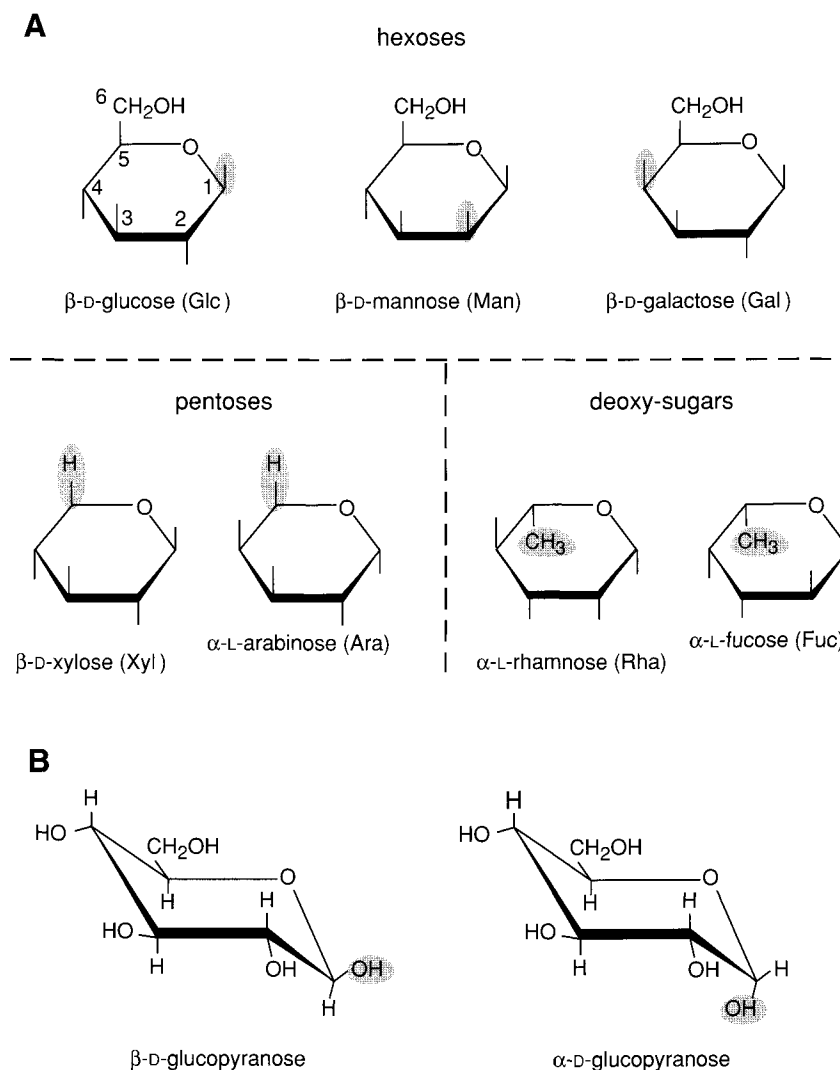


FIGURE 2-2 (A) Structures of sugars commonly found in plant cell walls. Hayworth projection formulae for pyranose ring structures are used for convenience. Note that some sugars in the wall occur in D configuration, whereas others occur in L configuration. Also, the position of the OH group on the anomeric carbon, carbon 1, determines the α vs β configuration. Arabinose and xylose are five carbon sugars, whereas others are six carbon sugars. Fucose and rhamnose are deoxy sugars. The Hayworth projection, although very convenient, is somewhat misleading in that the molecule appears planar with OH groups above and below the ring. Such is not the case. Due to the tetrahedral arrangement of bonds about the carbon atoms, the pyranose ring exists in the thermodynamically more stable chair conformation and the OH groups tend to lie equatorially rather than axially. (B) β -D-glucopyranose and, for comparison, α -D-glucopyranose are shown in the chair form as well.

after the sugar that forms the backbone; the preceding prefix denotes the sugar(s) that is present in the side chain. For example, xyloglucan is a heteropolymer where the backbone is composed of glucose residues with side chains of xylose.

3.1.1.1. Cellulose

Cellulose is a homopolymer of β -D-glucopyranose subunits linked by the (1 \rightarrow 4) bond. Thus, it is a β 1,4-glucan. Cellulose is present in cell walls of all plants, and some bacteria, and is the most abundant natural polysaccharide on earth. The stereochemistry imposed by the β 1,4-glycosidic linkage creates a linear glucan chain in which every other glucose residue is rotated 180° with respect to its neighbor (Fig. 2-3). Several cellulose chains come together to form a crystalline or paracrystalline lattice, which is stabilized by both intrachain (intramolecular) and interchain (intermolecular) hydrogen bonds. Intrachain hydrogen bonds are believed to form between the ring oxygen of one residue and the hydrogen of the C3 hydroxyl of a neighboring residue. Interchain hydrogen bonds occur between hydroxyl and oxygen atoms on adjacent chains. Additional stability is provided by van der Waal's forces. Several dozen such chains lie against each other and are bonded together to form what is known as a **cellulose microfibril**, or simply fibril. Most of the hydroxyl groups in a cellulose chain are used up in these bondings, resulting in a straight macromolecule of considerable strength and remarkable inertness. Cellulose is one of the most strong and durable natural materials.

Microfibrils in primary walls of vascular plants are often composed of ~ 36 β -glucan chains; some chains have as few as ~ 500 glucose residues, whereas others have ~ 2500 to 4500 residues. Within a microfibril, the individual chains may be staggered with more or less overlap with neighbors. Under the electron microscope, fibrils appear ~ 5 – 15 nm in diameter and up to several micrometers long (Fig. 2-4). In contrast, cell walls of some green algae (e.g., *Valonia*) have much bigger fibrils (30 nm in diameter), containing up to 1200 individual chains and typically $\sim 14,000$ glucose residues per chain.

Cellulose fibrils have both crystalline regions in which the individual chains are precisely arranged and amorphous regions where they are not. However, because of the presence of crystalline regions, cellulose appears birefringent under polarized light (for birefringence, see Box 2-1).

3.1.1.2. Noncellulosic Polysaccharides

Other polysaccharides in parenchyma cell walls are divided into pectins and hemicelluloses. A functional

definition of pectins is the wall fraction that is extracted by aqueous solutions of a chelating agent (e.g., ethylenediaminetetraacetic acid or EDTA, which binds divalent cations such as Ca^{2+}) or hot (70°C) dilute acid. In contrast, hemicelluloses are the fraction extracted subsequently by dilute to strong alkali (up to 4 M KOH at 25°C). The residue left in the cell wall after alkali extraction is referred to as α -cellulose, a term used in paper industry. It is worth noting that α -cellulose consists almost exclusively of β ,1-4-linked cellulose!

3.1.1.2.1. Pectins Pectins are a heterogeneous mixture of polysaccharides that are rich in uronic acids, particularly galacturonic acid. Uronic acids have a carboxyl, instead of an alcohol, function at C6 (see Fig. 2-5A). Several types of polysaccharides are extracted in the pectin fraction. They include polygalacturonans (PGAs) and rhamnogalacturonans (RGs), which are acidic polysaccharides and sometimes referred to as uronans. The others, such as arabinans, galactans, and arabinogalactans, are neutral polysaccharides. These various polymers may occur separately in the wall (*in muro*); but more likely, they occur interconnected and represent more or less discrete regions in an otherwise continuous matrix.

PGAs are relatively simple molecules; they are composed almost exclusively of D-galacturonic acid (GalA) residues, which are α 1,4-linked (see Fig. 2-5A). PGA is an unbranched homopolymer of GalA subunits. In contrast, RGs are highly complex and branched heteropolymers of α -L rhamnose and GalA. Based on the backbone structure and the nature of side chains, two types of RGs are recognized. In RG I, the backbone is composed of many repeats of a disaccharide unit, consisting of rhamnose and GalA (Fig. 2-5B). The bond linking the C1 of rhamnose to GalA is $\alpha(1 \rightarrow 4)$, whereas that linking C1 of GalA to rhamnose is $\alpha(1 \rightarrow 2)$. The backbone is quite long, because even after extraction from the wall by endopolygalacturonase, the degree of polymerization (DP) may still be ~ 2000 . Moreover, about half the rhamnosyl residues are further linked at C4 to a variety of side chains, predominantly arabinans, galactans, and arabinogalactans (see later), to yield some of the most highly branched and complex polysaccharides known. The PGA and RG I occur interconnected. Long stretches of relatively "smooth" regions of PGA may be followed by stretches of branched or "hairy" RG I.

RG II is a different type of molecule. Compared to RG I, it is a much smaller polymer with a DP of about 60. GalA and rhamnose are the most abundant constituents, but various other sugars (e.g., arabinose,

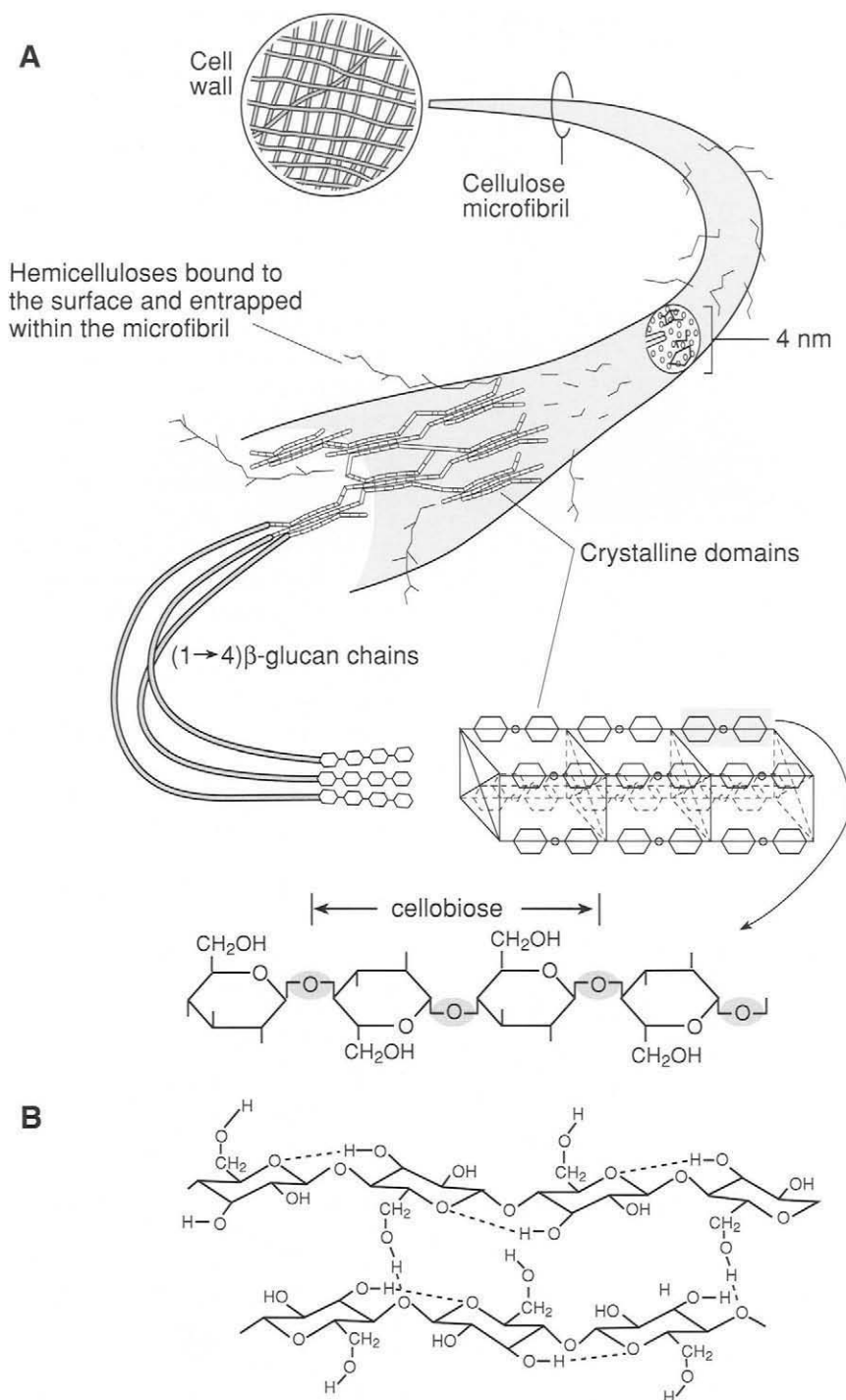


FIGURE 2-3 Structure of a cellulose fibril. (A) Cellulose consists of long chains of β -glucose units linked by β (1 \rightarrow 4) glycosidic bonds (shaded), with alternating units rotated 180° with respect to their neighbor. This means that the repeating unit is cellobiose, not glucose; also, the polymer forms a straight chain. Several cellulose chains are linked together to form a cellulose fibril, which is remarkably strong (tensile strength) and at the same time highly nonreactive. Most of the fibril has cellulose chains in a precise three-dimensional order and thus shows crystalline properties; other parts are amorphous or noncrystalline. From Taiz and Zeiger (1998). (B) Two (1 \rightarrow 4) β -glucan chains with intrachain and interchain hydrogen bonds.

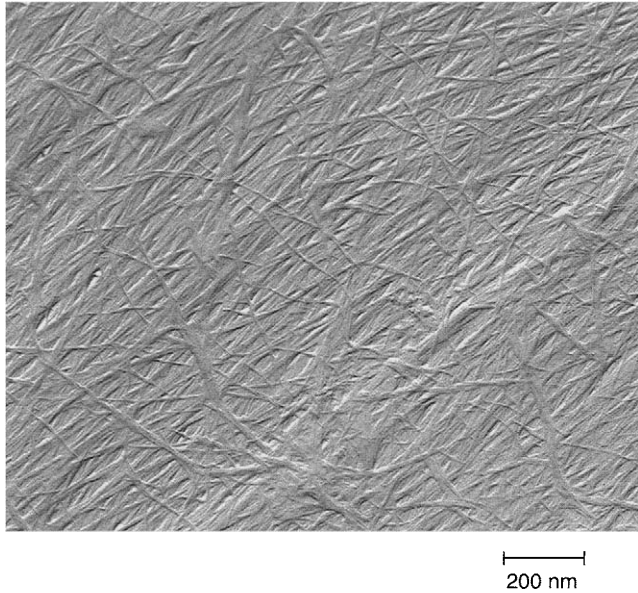


FIGURE 2-4 Cellulose microfibrils in the primary cell wall of *Zinnia elegans* cells in culture. Fibrils show random orientation and are approximately 6 nm in diameter; their length cannot be gauged in this photograph. For this photograph, the cells were cleaned in acid, leaving only the cellulose component of the cell wall; then the walls were shadowed with platinum and carbon, and the cellulose removed with strong acid leaving only the replica of the fibrils behind. Photograph courtesy of Mark Grimson and Candace Haigler, Department of Biological Sciences, Texas Tech University, Lubbock, Texas.

galactose) and uronic acids (e.g., glucuronic acid or GlcA), as well as some unusual glycosyl residues (e.g., apiose, methylfucose, methylxylose), occur in the backbone or in the side chains. Some fragments released by partial hydrolysis of RG II are shown in Fig. 2-5C.

In addition to the acidic polysaccharides, PGAs and RGs, pectins from different plant sources contain variable proportions of long and branched chains of arabinans, galactans, and arabinogalactans. As mentioned earlier, these polymers occur as side chains of RG I, but they may also occur relatively freely in the wall matrix. Arabinans are highly branched molecules, with a backbone of α 1,5-linked arabinose residues and side chains of single arabinose residues linked by α (1 \rightarrow 2) or α (1 \rightarrow 3) bonds. Galactans are essentially linear, β 1,4-linked homopolymers of galactose. Arabinogalactans have a backbone of β 1,4-linked galactose residues with frequent side chains of arabinose or arabinans linked by α (1 \rightarrow 3) bonds (Fig. 2-6).

The uronans, PGAs and RGs, carry many negatively charged carboxyl groups, which may be esterified to methyl groups, to other pectins and noncellulosic polysaccharides, and to phenolic compounds, such as ferulic acid. These groups also form ionic interactions with

cations, and with basic amino acids in cell wall proteins. Because PGAs form helical chains with an even spacing of carboxyl groups, they are particularly important in forming gels. In the wall, two antiparallel PGA chains can condense by cross-linking with Ca^{2+} to form egg box-like "junction zones" (Fig. 2-7). The ionization status of uronans, especially PGAs, is markedly affected by extracellular pH and thus provides a ready means to alter the physical properties of this matrix.

3.1.1.2. Hemicelluloses Hemicelluloses represent the neutral sugar fraction extracted from the walls by alkali treatment, following the extraction of pectins. In plant tissues consisting of cells with primary walls only (not secondary walls, such as wood), they are represented mostly by xylans, xyloglucans (XGs), and mixed linkage glucans. (Many other hemicelluloses, such as glucomannans in secondary walls of conifer woods and mannans and galactomannans in storage tissues of some seeds are known. They are not considered here.) These polymers are not extracted by aqueous solutions of chelating agents or by dilute acid because they are strongly hydrogen bonded to cellulose fibrils and require strong alkali to break those bonds.

The relative proportions of xylans and XGs vary in primary walls of different taxa. In cell walls of conifers, dicots, and many monocots, XGs occur in abundance with only small amounts of xylans. The reverse is true in primary walls of other monocots, particularly grasses, which also show substantial amounts of mixed link glucans.

3.1.1.3. Xyloglucans

The backbone in XGs is a chain of D-glucose units linked β 1,4, as in cellulose, although it is much shorter. The backbone carries single xylopyranosyl substituents linked to it by α (1 \rightarrow 6) linkage. These substituents usually occur in a precise and orderly manner. Three successive glucose residues, out of four, in the backbone are linked to xylose; the fourth glucose residue is free. The backbone, therefore, can be considered to be a general repeat of Glc_4Xyl_3 subunits. Many of these xylosyl units are further substituted by galactose or by galactose, as well as fucose (Fig. 2-8A). The result is a glucan backbone with mono-, di-, or trisaccharide side chains at periodic intervals. Glucose residues in the backbone may also be linked to other sugars, e.g., arabinose, directly. The existence of the structural subunit Glc_4Xyl_3 was elucidated by the use of a purified *endo*- β (1 \rightarrow 4)-D-glucanase, which specifically hydrolyzes the glycosidic bond following the unsubstituted glucose residue (shown by open arrows), thus releasing the subunit oligosaccharides.

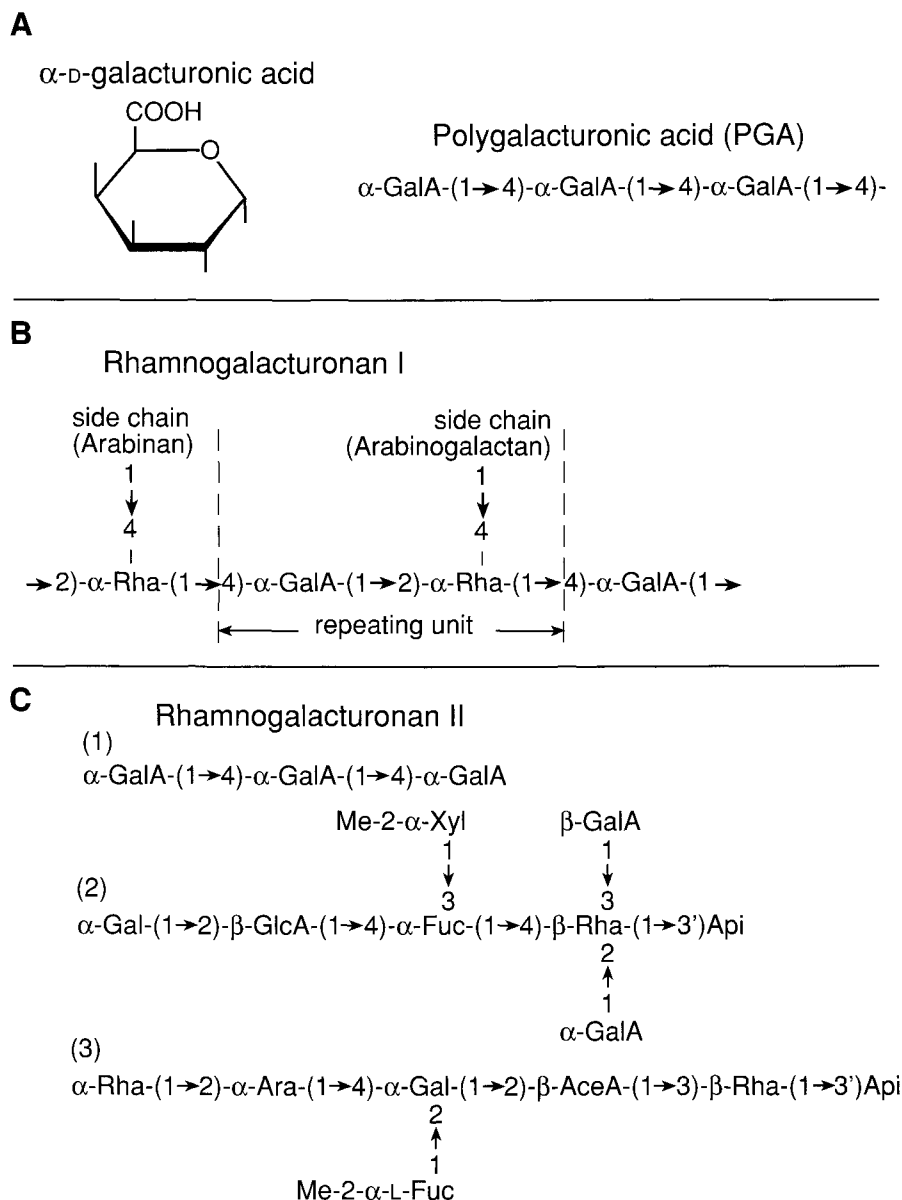


FIGURE 2-5 Acidic pectic polysaccharides. (A) Structures of galacturonic acid and PGA. Uronic acids have a carboxyl group at C6. PGAs are homopolymers of galacturonic acid-linked α 1,4. (B) Rhamnogalacturonans I (RG I) are heteropolymers of repeating (1 \rightarrow 2)-linked disaccharide units of α -L-rhamnosyl-(1 \rightarrow 4)- α -D-GalA. Many rhamnose units carry side chains, thus forming a highly branched polymer. Adapted from Carpita and Gibeaut (1993). (C) Three oligosaccharide fragments released by partial acid hydrolysis of RG II. AceA, aceric acid; Api, apiose are rare sugars. From Brett and Waldron (1996) with kind permission from Kluwer.

3.1.1.4. Xylans

Xylans are linear chains of β 1, 4-linked xylose units. The backbone carries several different types of substitutions, but mainly single arabinose units at C2 or C3 and, less frequently, single glucosyluronic acid (GlcA) units at C2 of some xylosyl units. GlcA substitutions impart an acidic nature to these otherwise neutral polysaccharides. Hence, they are sometimes referred to as glucuronoarabinoxylans (GAXs) (Fig. 2-8B).

3.1.1.5. Mixed-Linkage Glucans

These are linear, unbranched polymers of D-glucose residues linked β (1 \rightarrow 4, 1 \rightarrow 3) (see Fig. 2-8C). The relative proportions of the two types of linkages and their distribution within the molecule are not random, but ordered. The (1 \rightarrow 4) linkages occur usually in blocks of two or three and are separated by (1 \rightarrow 3) linkages, which occur singly. As in the case of xyloglucans, the structural regularity of mixed linkage glucans was

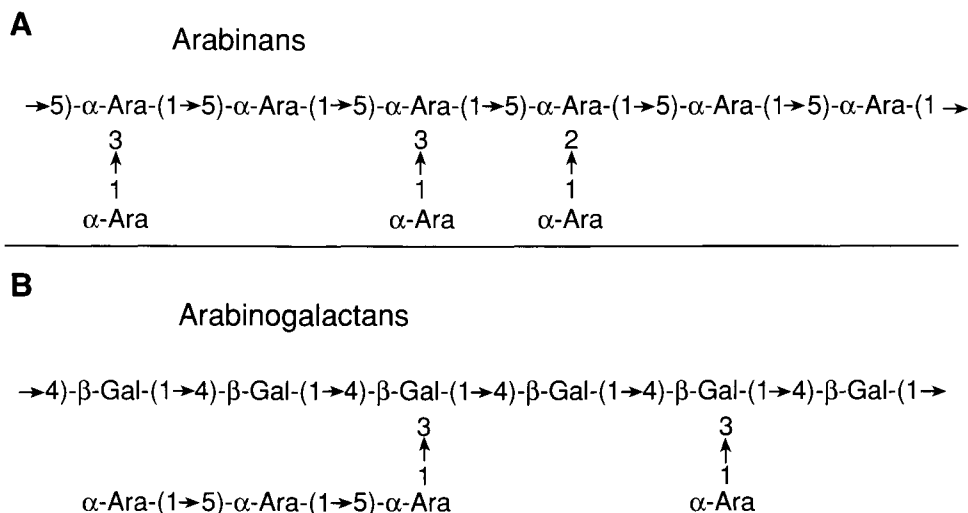


FIGURE 2-6 Structures of some neutral polysaccharides in pectin fraction: (A) arabinans and (B) arabinogalactans.

detected with the help of an enzyme with a specific cleavage site. The enzyme was a highly specific β -glucanase ("lichenase") from the bacterium *Bacillus subtilis*. This enzyme hydrolyzes $\beta(1 \rightarrow 4)$ glycosidic bonds, but only if they immediately follow a $\beta(1 \rightarrow 3)$

glucosyl link (shown by open arrows). The enzyme thus releases glucooligosaccharide fragments, where all residues are linked $\beta(1 \rightarrow 4)$, except for the terminal residue at the reducing end, which is $\beta(1 \rightarrow 3)$ linked.

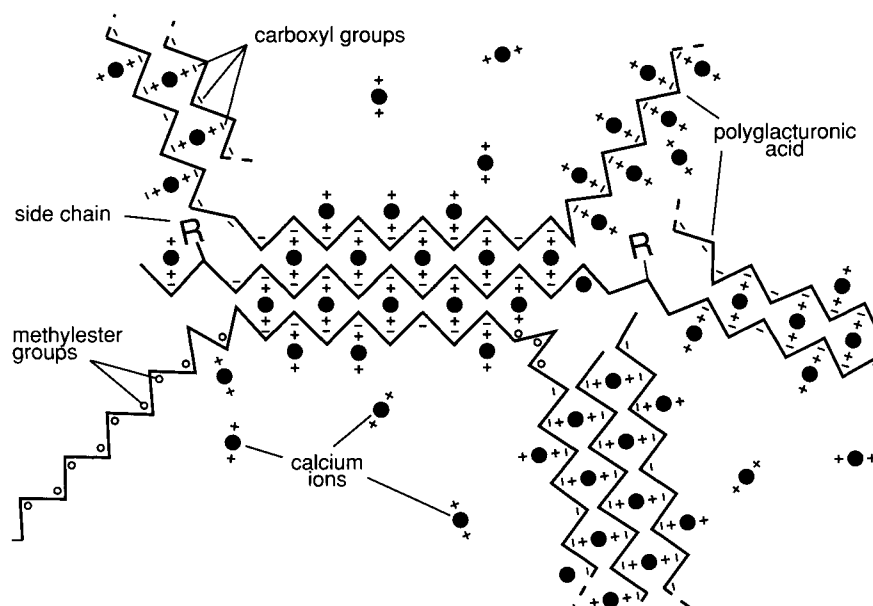


FIGURE 2-7 Three antiparallel chains of PGA cross-linked by Ca^{2+} to form "egg box" junctions. Regions esterified to methyl groups and free anions are also shown. From Brett and Waldron (1996) with kind permission from Kluwer.

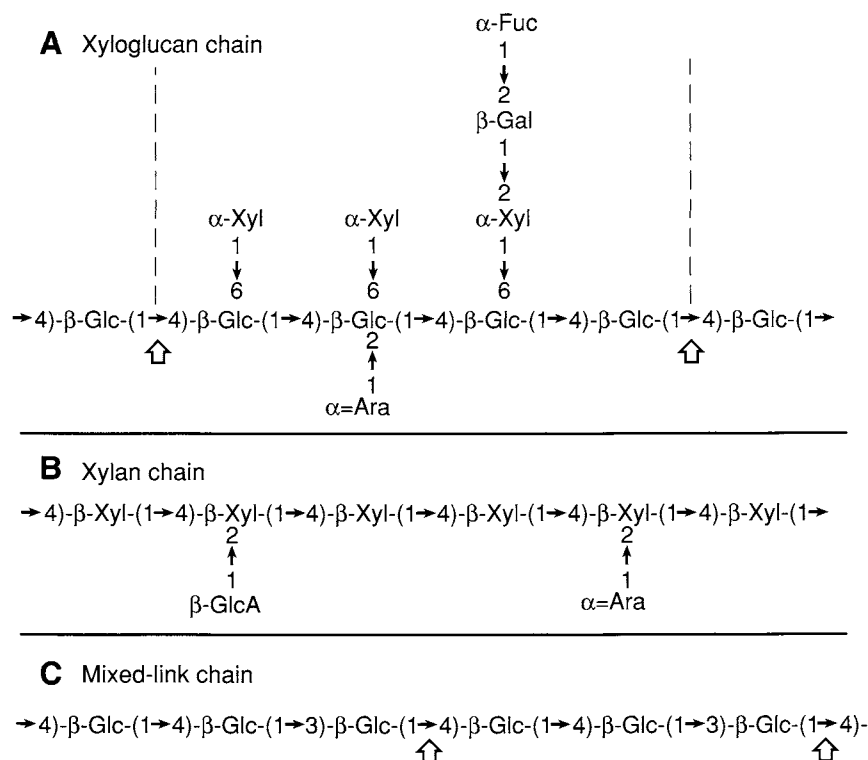


FIGURE 2-8 Structures of xyloglucans (XGs) (A) and xylans (B). Adapted from Brett and Waldron (1996) with kind permission from Kluwer. (C) Mixed-linkage glucans. These hemicelluloses occur in close association with cellulose fibrils. From Reid (1997).

BOX 2-1 PROBING WALL STRUCTURE

THE STRUCTURE OF PLANT cell walls has been investigated by a variety of techniques. A great deal of early information on the structure and orientation of cellulose fibrils in primary and secondary walls was provided by X-ray crystallography and polarization microscopy. X-ray crystallography by R. D. Preston at University of Leeds confirmed the precise spacing of glucose residues in a chain—the glycosidic oxygen bridges occur at intervals of 5.15 Å. It also provided clear evidence that cellulose chains within a fibril show a precise three-dimensional order, thus establishing the crystalline nature of this polysaccharide. Many crystalline materials have the property of rotating the plane of polarized light. If a polarizer and analyzer are set in a microscope such that no light comes through the eye piece, a state known as the extinction point, and then a crystalline material is introduced at the specimen stage, it turns the plane of polarized light and light shines through, a phenomenon known as birefringence. If the polarizer is rotated by a certain angle, extinction can be reached again. The angle of rotation provides a clue to the orientation of the crystal. Cellulose alone among the wall constituents has a crystalline structure and shows birefringence. Birefringence of cellulose fibrils is greatest if the polarized light strikes the fibrils at right angles to their length, it is least when it strikes the fibrils end on. Using the angle of rotation technique, Irving Bailey at Harvard University, Cambridge, MA, and A. Frey-Wyssling at the Swiss Federal Institute of Technology, Zurich, Switzerland, deduced accurate conclusions about the orientations of cellulose fibrils in many different types of walls (Fig. 2-9A). Visualization of cellulose fibrils was not possible until the advent of transmission electron microscopy (TEM) in the 1950s and 1960s; and Kurt Mühlethaler also at the Swiss Federal Institute of Technology, Zurich, Switzerland was one of the first to publish photographs of cellulose fibrils. Walls could be selectively extracted for pectins, or hemicelluloses, or proteins, and the extracted wall could be shadow casted and studied under the TEM. In the 1980s, techniques were evolved for very faithful preservation of wall structure by rapid freezing under

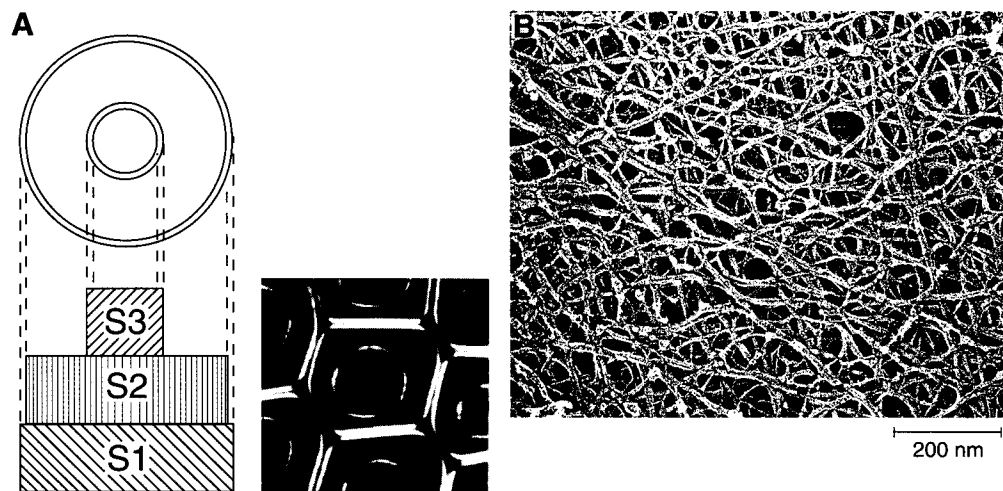


FIGURE 2-9 (A) Schematic illustration of the orientation of cellulose fibrils in secondary wall layer 1 (S1), layer S2, and layer S3 (left) and a light micrograph of a cross section of xylem tracheids under polarized light (right). In the cross section, the S1 and S3 appear birefringent, but the S2 appears dark because the direction of polarized light parallels that of cellulose fibrils in the S2 layer. From Kerr and Bailey (1934). (B) The Cell wall of onion leaves was extracted to remove most of pectins, frozen under pressure, ice was sublimated, and then a carbon replica was made and shadow casted before examination under on electron microscope. Courtesy of Maureen McCann, John Innes Center, Norwich, UK.

pressure, followed by fixing and preliminary embedding of the material under very low temperature. The photograph of a cell wall from an onion leaf in Fig. 2-9B was obtained after combination of the two techniques of selective extraction and rapid freezing. Antibodies against specific fragments of wall polysaccharides allow visualization of specific sugars and uronic acids in the walls. The rapid freezing and immunocytochemical techniques are described in greater detail in Box 2-2.

Extraction of wall polysaccharides and their chemical characterization had been done since the 19th century, but real progress in understanding how various sugars and uronic acids are linked together in complex polysaccharides in primary walls was not possible until the advent of gas-liquid chromatography (GLC) and high-pressure liquid chromatography (HPLC) for the separation of minute amounts of chemicals and mass spectroscopy (MS) for their unambiguous identification (see Box 5-1 in Chapter 5). These techniques became available in the 1960s and 1970s. Peter Albersheim and colleagues, then at the University of Colorado, Boulder, CO, devised suitable derivatization techniques for GLC of carbohydrates and published the first papers on how sugars and uronic acids are linked together in complex carbohydrates; they proposed the first model of primary wall structure in 1973. For much of their work, they used suspension cell cultures from sycamore maple (*Acer pseudoplatanus*), which provided a ready source for sufficient amounts of a reasonably uniform plant material. Linkage study does not give the sequence of monomers in an oligosaccharide chain, and sequencing, as is routine for DNA and proteins, is not done. There are, however, some "restriction glycanases," enzymes produced by plant pathogens, which cleave at specific linkages. These enzymes, as indicated earlier, have proved valuable in the elucidation of the subunit structure of xyloglucans and mixed linkage glucans.

Other approaches of study include X-ray diffraction, ^1H - and ^{13}C – NMR, and computer simulations. Polarized infrared (IR) and Fourier transform IR (FTIR) microspectroscopy are used to observe the relative amounts of matrix polysaccharides and proteins and how the different polymers are oriented and re-arranged in the walls.

In the 1960s, Derek Lamport at MSU-DOE Plant Research Laboratory, East Lansing, MI, showed that glycoproteins, such as extensin, form an integral part of wall structure. Since then, many other types of glycoproteins have been shown to be present in the cell walls. Genes for the protein moieties of these glycoproteins have been cloned and sequenced, and their cDNAs are used for tissue printing analyses. A tissue print indicates the presence of the mRNA encoding the protein. James Varner and colleagues at Washington University, St. Louis, MO, pioneered the application of tissue print technique to study wall proteins. These studies indicate that the wall structural proteins are synthesized in specific cell types at certain times in their development.

3.1.2. Proteins

Many different types of proteins, both structural and enzymatic, occur in cell walls. The structural proteins are mostly conjugated with short chains of oligosaccharides, and hence are glycoproteins. The most abundant of these glycoproteins are the ones that are rich in an unusual amino acid, hydroxyproline, which is absent from proteins in the protoplast. The presence of hydroxyproline in a fraction marks it as coming from the wall. Hydroxyproline-rich glycoproteins (HRGPs) are among the most investigated wall proteins; others include proline-rich glycoproteins (PRPs) and glycine-rich glycoproteins (GRPs).

3.1.2.1. HRGPs

The most common HRGP is extensin. Many different types of extensins are known from dicot and mono-

cot walls, particularly those associated with vascular tissues, cambial cells, and cells in culture (Table 2-1).

Extensins in dicots are rich in serine and hydroxyproline and usually show a pentapeptide repeat motif, Ser-(Hyp)₄, separated by an average of about six amino acids, among which valine, tyrosine, lysine, and histidine are common. Most of the Hyp residues are glycosylated with short, tri- or tetrasaccharide chains of arabinose residues (Fig. 2-10A). Some of the Ser residues are glycosylated with a single galactosyl unit. Monocots have somewhat different versions of extensins. They are also less glycosylated (see Table 2-1).

Extensins are basic proteins with isoelectric points (pI) of ~10 due to their high lysine content. In solution, they often assume a secondary helical structure, which makes them appear as kinked rods in electron micrographs (Fig. 2-11). Tyrosine residues in the molecule are able to cross link with each other. Thus, they

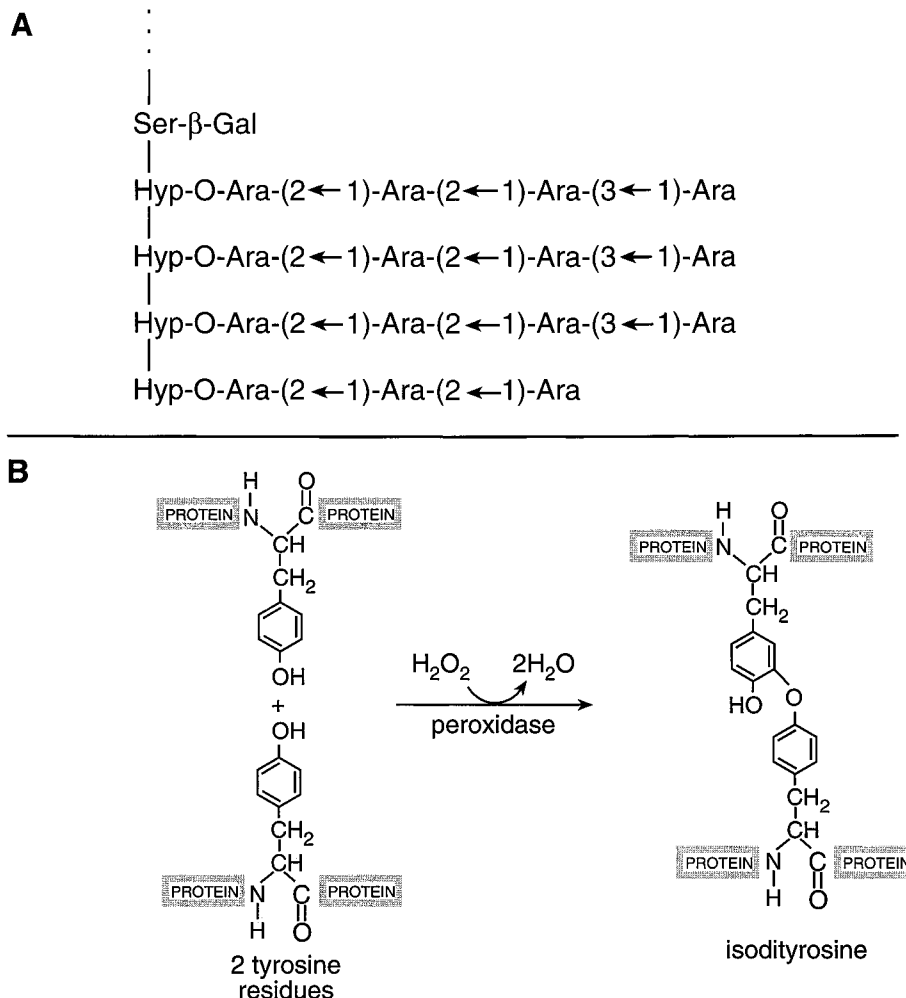


FIGURE 2-10 Extensin. (A) Ser-(Hyp)₄ subunit of extensin with oligosaccharide side chains. (B) Isodityrosine linkage between two extensin monomers. From Brett and Waldron (1996) with kind permission from Kluwer.

TABLE 2-1 Common Wall Proteins, Their Protein and Sugar Contents, and Their Abundant Amino Acids^a

Protein class	Protein (%)	Sugar (%)	Abundant amino acids
Extensins (dicot)	~ 45	~ 55	Hyp, Ser, Lys, Tyr, Val, His
"Extensins" (monocot)	~ 70	~ 30	Hyp, Thr, Ser, Pro, Lys
AGPs	2–10	90–98	Hyp, Ser, Ala, Thr, Gly
Solanaceous lectins	~ 55	~ 45	Hyp, Ser, Gly, Cys
GRPs (dicot)	~ 100	~ 0	Gly
GRPs (monocot)	~ 100	~ 0	Gly
PRPs	~80–100	0–20	Hyp, Pro, Val, Tyr, Lys

^aFrom Showalter (1993).

can form intramolecular bridges and also have the capacity to link two extension molecules together and form an extensin network (Fig. 2-10B). In walls that have been extracted with HF to remove all polysaccharides, including cellulose, a protein network still remains and can be visualized under the electron microscope.

It was once assumed that extensins had a role in cell extension. That role is no longer considered valid. Although the precise function of extensins is still unclear, they are now believed to serve a stabilizing or

reinforcing role in the wall of cells that have stopped elongating.

Other hydroxyproline-rich glycoproteins include some lectins in members of the potato family (Solanaceae) and some arabinogalactan proteins (AGPs) (see Table 2-1). Lectins are special glycoproteins produced by plants for defense against herbivores and plant pathogens (for more on lectins, see Chapter 20). AGPs occur as soluble secretions in many tissues, including ones in pistils of flowers and in suspension cell cultures. The carbohydrate moieties of these proteins probably play a role in cell-cell recognition. There is substantial evidence for such a role in for AGPs, pollen tube-pistil interaction. These proteins do not seem to be involved in wall structure and hence are not considered here.

3.1.2.2. PRPs and GRPs

Other structural glycoproteins include proline-rich proteins and glycine-rich proteins (Table 2-1). PRPs are more highly expressed in the later phases of cell growth and are believed to lock the extensin network, and cell shape, in place. GRPs seem to constitute a diverse group, which may be expressed in cells that show lignification, either in the course of normal differentiation, as in xylem cells, or after wounding or pathogenesis.

3.1.2.3. Enzymes

Cell walls also harbor many enzymes. The full extent of enzymatic proteins in walls is still unknown and their specific location in, or association with, the wall is a matter of conjecture. Many of them appear to be soluble proteins because they are extracted by aqueous solvents. One major class of enzymes is involved in modifications of wall architecture by hydrolyzing one or another wall component (Table 2-2). These enzymes play important roles in cell growth during elongation

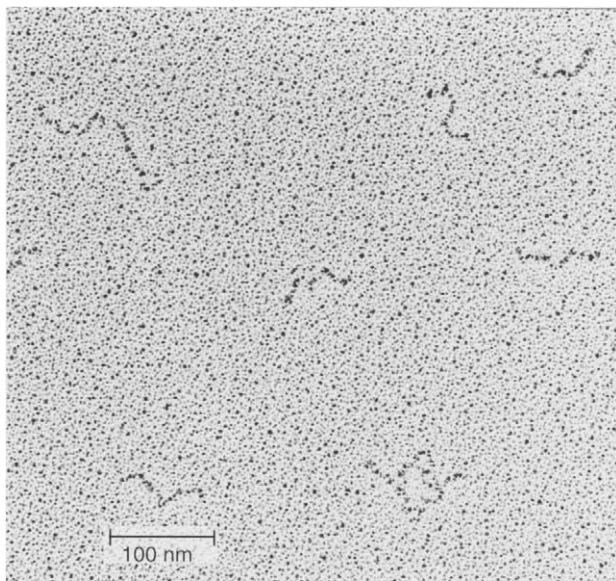


FIGURE 2-11 Extensin molecules as seen under a transmission electron microscope. Individual molecules appear as kinked ~80-nm rods; the complex on lower right shows a cross-linked extensin oligomer. For this electron micrograph, extensin was extracted with a salt solution from carrot root tissue, purified, and sprayed onto a mica surface. The sample was rotary shadowed at a low angle with platinum/carbon, a replica was made from the shadowed preparation, and the replica was examined in the EM. Courtesy of Andrew Staehelin, University of Colorado, Boulder, CO.

TABLE 2-2 Major Enzymes Responsible for Modifications in Wall Architecture

Enzyme	Postulated action
Endo- β -1,4-glucanases (also referred to as "cellulases")	Break the β -1 \rightarrow 4 link in the glucan backbone of xyloglucans (XGs), not crystalline cellulose
Xyloglucan endotransglycosylases; also called endoxyloglucan transferases	Cleave the β -1,4-link in XGs at specific places, the glycosidic link before the unsubstituted glucose residue, also thought to reattach XG segments
Expansins	Thought to break hydrogen bonds between cellulose fibrils and XGs
Pectin methylesterases	Hydrolyze methyl ester of PGA and thus create free carboxyl groups that can link with Ca^{2+} ions and stabilize two PGA chains
Polygalacturonases	Break the α (1 \rightarrow 4) bonds between GalA units in PGA and lead to solubilization of pectins

or expansion of stems, roots, and fruits; softening of the wall (pulp) during fruit ripening; and abscission of leaves, fruits, and other organs. They are covered in more detail in Section III and in Chapters 15, 17, and 20. Cellulose synthase, the enzyme responsible for cellulose synthesis, is thought to be an integral plasma membrane protein with cytoplasmic domains and is covered later in this chapter. Various other synthases, transferases, hydrolases, peroxidases, and invertases that modify other substrates are also reported in the walls. Yet another class of enzymes occur in the walls, e.g., chitinases, (1 \rightarrow 3) β -glucanases, which are involved in plant defense against pathogens, such as bacteria and fungi.

3.1.3. Phenolic Substances

Phenolic substances are aromatic compounds with at least one phenol group, i.e., a benzene nucleus with a hydroxyl group. Plants produce hundreds of different kinds of phenolic substances (see Table 2-3). Some common phenolics such as anthocyanins and hydrolyzable as well as condensed tannins accumulate in the cell vacuole. Some other phenolic substances are deposited in the walls. The most well-known phenolic substance that occurs in cell walls is lignin, but, as mentioned earlier, it is deposited in certain cell types after cell growth has ceased. Phenolic substances that are deposited in walls of parenchyma cells include ferulic acid, *p*-coumaric acid. These phenolics occur bonded to various sugars in the cell walls of parenchyma cells and are particularly abundant in cell walls of grasses. Ferulic acid is important because it is known to form diferulate links between arabinose or galactose residues in side chains of pectins and may serve to cross-link them (Fig. 2-12). The extraction of

pectins from these walls (type 2 walls, see below) is, therefore, much more difficult.

3.2. Proportions of Wall Constituents Vary among Taxa and in Different Wall Layers of the Same Cell

The proportions of various wall constituents, polysaccharides, proteins, and phenolic materials vary considerably among different taxa and wall layers. Based on relative distribution of these materials and their three-dimensional structure, two types of primary walls have been recognized. Type 1 walls, present in gymnosperms, dicots, and most monocots (other than grasses), have about 80–90% polysaccharides, about 10–20% proteins, and little, if any, phenolic materials. Among the polysaccharides, cellulose and XGs occur in about equal proportions (about 25% each), pectins account for about 30%, and the amounts of xylans are very limited. Type 2 walls, present in grasses (Poaceae), have ~20% or so phenolic

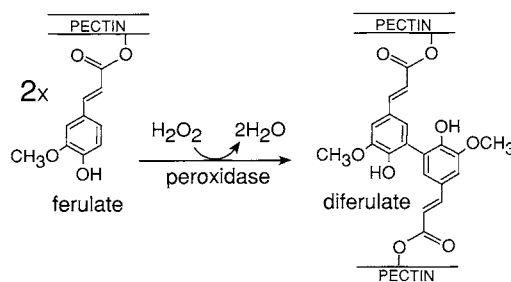


FIGURE 2-12 Cross-linking of pectins by ferulic acid. From Brett and Waldron (1996) with kind permission from Kluwer.

substances (other than lignin), more xylans, and mixed linked glucans, relatively little XGs, and much less pectins.

The proportions of pectins and cellulose vary in different wall layers of the same cell. The middle lamella is often rich in pectins, especially unesterified PGAs, which are bonded to Ca^{2+} , and low in cellulose, whereas the reverse is true in primary walls. The immunogold labeling technique (see Box 2-2) shows that extensin is present through most of the primary wall, but is conspicuously low in amount or absent from the middle lamella.

The secondary walls are usually richer in highly ordered layers of cellulose and are lower in pectins than primary walls or middle lamella. As a result, the secondary walls show strong birefringence under polarized light, primary walls less so, whereas the middle lamella appears isotropic. Secondary walls also differ in their hemicellulose composition among different taxa. For example, xylem cell walls in conifer woods have a high proportion of glucomannans and much less xylans, whereas those in hardwoods have lesser amounts of glucomannans and much higher amounts of xylans.

3.3. Molecular Architecture of Primary Cell Walls

Wall constituents are like brick and mortar; how they are put together to serve the various functions of a living plant cell involves design and architecture. Models of wall structures are based on data from enzymatic digestions, or sequential extractions, of specific wall constituents and chemical analyses coupled with polarization optics or electron microscopy and immunocytochemistry (see Box 2-1). These studies have been done on a few selected plant systems, such as sycamore suspension cell cultures, onion leaves, grass leaves, and pea epicotyls, and although a great deal of information has become available, the reader must understand that these models are not necessarily applicable to all plant cell walls.

3.3.1. Wall as a System of Interconnected Networks

3.3.1.1. Cellulose–Hemicellulose Network

In type 1 walls, XGs and small amounts of GAXs hydrogen bond to cellulose fibrils. They are thought to coat cellulose fibrils with a monolayer of hemicellulose (Fig. 2-13A). As mentioned earlier, in type 1 walls, XGs and cellulose occur roughly in a 1:1 proportion. However, XG is only able to form hydrogen bonds on one side of the molecule because of limitations imposed by the side chains, especially di- and trisaccharide chains.

Hence, only part of XG can be bound to cellulose fibrils; the remainder is thought to span the gaps between fibrils, forming tethers that hold the cellulose fibrils in place. Such tethers or cross-links are seen in electron micrographs of walls, which have been selectively extracted for pectins (Fig. 2-13B). The length of XGs in these electron micrographs varies between 20 and 40 nm, which also reflects the gap between cellulose fibrils; however, isolated molecules of XG are much longer (average about 200 nm), sufficient to span the gap between fibrils and to hydrogen bond to two microfibrils at each end. Sections of XGs in the interfibrillar space may bind to each other, as well as to other matrix components.

In type 2 walls, GAXs and $\beta 1,3, \beta 1,4$ -mixed link glucans bind to cellulose fibrils similarly. Here, also, the degree of hydrogen bonding to fibrils may be limited by the extent and nature of side branches in GAX and the kinks imposed by the $\beta 1,3$ link in mixed linkage glucans.

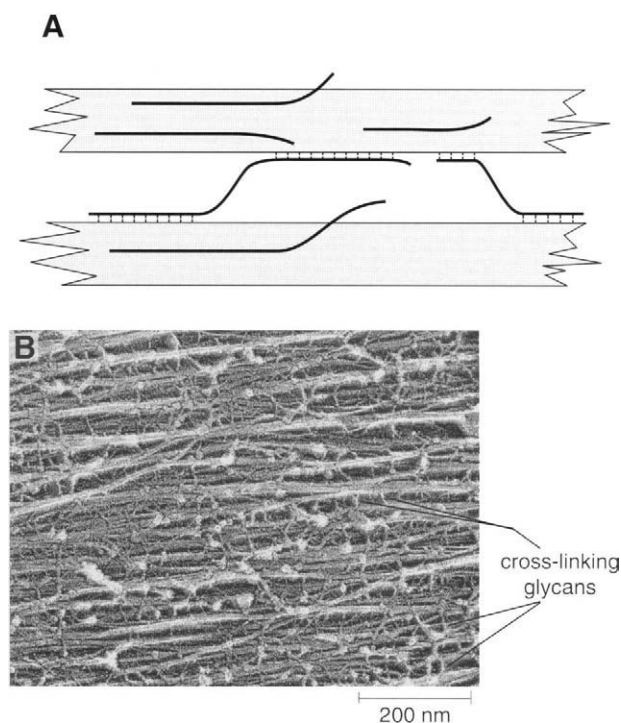


FIGURE 2-13 (A) Schematic model of a cellulose fibril and attached xyloglucan molecules. The orientations of XG backbone and the side chains are hypothetical. (B) An electron micrograph of a wall from an elongating carrot cell in culture showing oriented cellulose fibrils and cross-linking glycan bridges. The bridges are 30 nm long and connect neighboring cellulose fibrils. For this photograph, wall fragments were extracted with an aqueous solution of a chelating agent and 1.0 M KOH before being rapid frozen and prepared for electron microscopy. Courtesy of Brian Wells and Keith Roberts, John Innes Center, Norwich, UK.

3.3.1.2. Pectin Network

The cellulose–hemicellulose network is embedded in the pectin network or pectic matrix. In type 1 walls, most of the pectins can be extracted from the cell walls without affecting the cellulose–hemicellulose network. Hence, it is thought that the two networks are independent of each other, and covalent bonds, if any, between them are few. The situation with respect to type 2 walls is less clear. These walls have proportionally less pectins; also, as mentioned before, they have large amounts of phenolic compounds in etherified linkage with arabinose or galactose residues in hemicelluloses and pectins, which makes their extraction difficult.

3.3.1.3. Protein Network

Glycoproteins in the wall, particularly extensins, form a third network. The extensin chains are believed to run through the thickness of the wall, like pins in a cushion, and have an orientation perpendicular to the plane of the plasmalemma and that of the cellulose layers.

3.3.2. Models of Primary Wall

Models of primary walls visualize these three networks as an interconnected system, but differ in the degree of covalent linkage between networks. In earlier models, all bonding was believed to be covalent, except that of XGs to cellulose microfibrils, which was hydrogen bonding. The more recent models visualize the networks as separable entities (Fig. 2-14). The pectic matrix may have no covalent linkage with the cellulose–XG network, it may simply be embedding it. Likewise, the protein, specifically extensin, network may have no covalent links with either the pectin or the cellulose–hemicellulose network. Nonetheless, the networks are physically intertwined and interdependent in the sense that if one network is affected, the other network may tend to compensate for it.

3.3.3. Roles of Various Constituents

The cellulose–hemicellulose network forms the structural framework for the wall and provides strength in both longitudinal and transverse planes. It also plays a central role in cell growth. As long as the hydrogen bonds holding the hemicelluloses to cellulose fibrils are in place, only very limited growth can occur. An essential part of cell growth is a breakage of these bonds and their reestablishment for the continuous requirement of strength and support. Because these two components are held together by hydrogen

bonds, factors that control the extent of hydrogen bonding are likely to be developmentally regulated. In this context, some data indicate that the extent and nature of substitutions in the backbone of GAXs, also the frequency of β 1,3 links in the mixed linkage glucans, change with the developmental state of a cell; they are higher in growing cells and decrease as a cell matures.

Pectins, other hemicelluloses, and proteins provide filler material and constitute the matrix of the wall. Pectins serve several important roles; they are not an “amorphous jelly.” They play a structural role in the cell wall. Binding of GalA units in PGA to Ca^{2+} results in calcium pectate, which is the cementing substance that keeps two cells together. Removal of calcium makes the cells fall apart. Unesterified GalA or GlcA units may also form ionic links with basic amino acids, such as lysine, in the wall proteins. The strength and firmness of the pectic network are also influenced by hydrophobic interactions between stretches of PGA that are methyl esterified. In type 2 walls, an increase in frequency of diferulate links can make the network more rigid. Structural and physical changes in pectins can occur independently of the cellulose–hemicellulose network. If cellulose synthesis is prevented in cells, pectins take on a more structural role. For instance, 2,6-dichlorobenzonitrile is a herbicide that specifically inhibits cellulose synthesis. Suspension cultured tomato and tobacco cells treated with dichlorobenzonitrile develop a tightly cross-linked pectin network, which becomes the main structural component of the cell wall.

Pectins also play a role in maintaining an ionic and pH balance in the extracellular environment, which in turn affects the porosity of the wall matrix. Changes in the ionization status of carboxyl groups, and the extent of methyl esterification or binding to other substances change the pH of the wall, which in turn affects the porosity of the wall matrix and movements of various macromolecules across the cell wall, as well as access of various enzymes to their substrates.

The exact role of extensins is still unclear. They may provide stability and interlocking once cell growth has ceased. Tissue print studies indicate that mRNAs of proline-rich proteins appear at an earlier stage and that mRNAs for extensin-type proteins appear near the maturation stage in the development of a cell. Accordingly, it is thought that PRPs may have a role in cementing the extensin network together. mRNAs of glycine-rich proteins are selectively expressed in cells that are destined to undergo lignification. Thus, it is believed that GRPs may serve to anchor the lignin polymers in the wall.

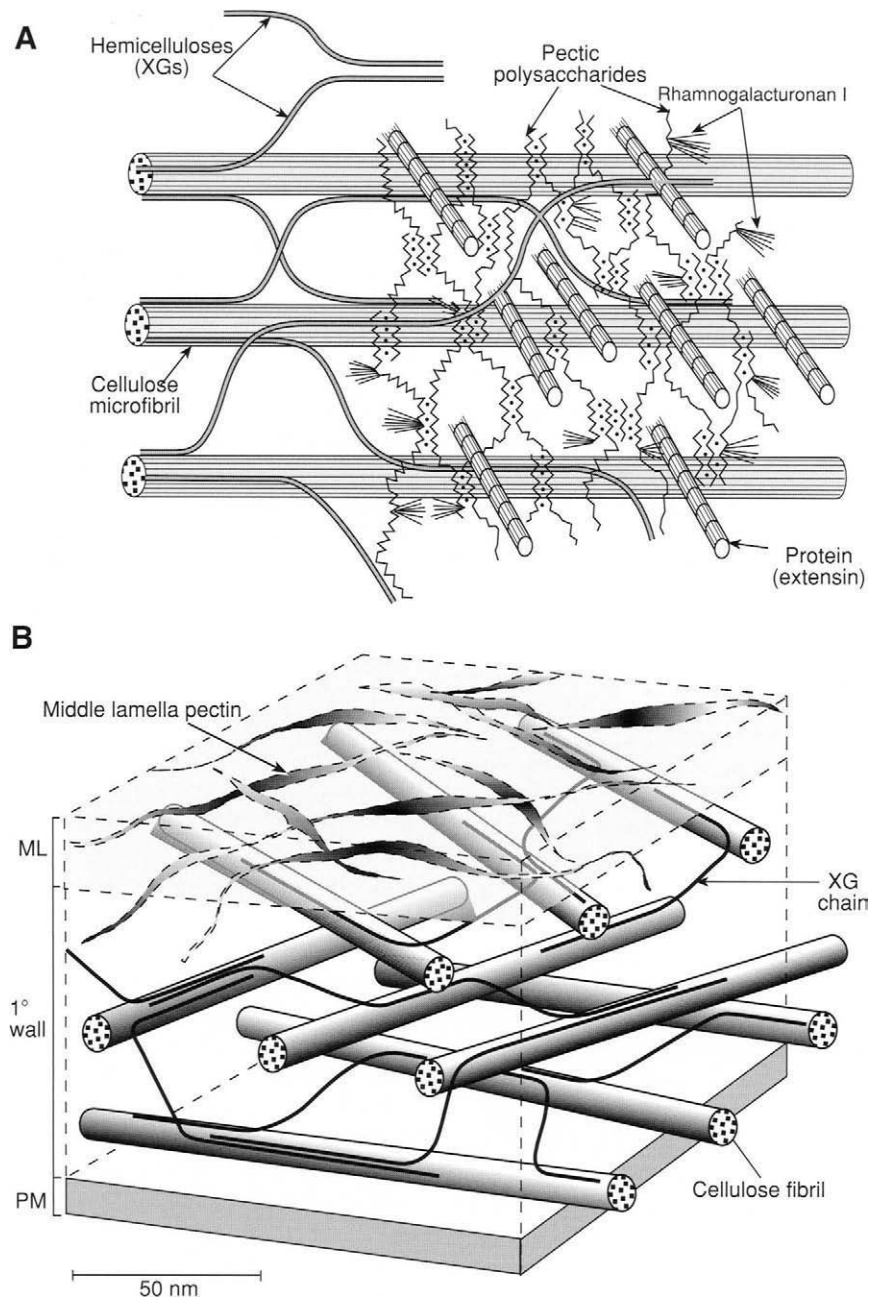


FIGURE 2-14 Major structural components and their likely arrangements in type 1 primary walls. (A) The cellulose–hemicellulose network, the pectin network, and structural proteins are shown as more or less separate entities. Hemicellulose (xyloglucan) chains are partly buried within and partly coat the surface of fibrils; they also tether neighboring fibrils. Pectins are shown with PGA junction zones interspersed with smooth and hairy regions of RG I. Proteins, such as extensins, are thought to run through the thickness of the wall, like pins in a cushion. Adapted from Brett and Waldron (1996) with kind permission from Kluwer. (B) A three-dimensional model of the wall showing only cellulose fibrils and xyloglucan (XG) chains. Fibrils are arranged in layers and, because a wall has a certain thickness, there may be many such layers in a wall (three such layers are shown in the primary wall, 1° wall). Within a layer, the fibrils run more or less parallel to each other and predominantly in one orientation. Hemicellulose chains are shown tethering neighboring fibrils in the same layer, as well as in layers above and below. The middle lamella (ML) is composed predominantly of pectins and relatively little cellulose. In this drawing, it is shown free of cellulose fibrils. PM, plasma membrane. Adapted from McCann and Roberts (1991).

3.4. Synthesis/Deposition of Wall Materials

The synthesis/deposition of various wall materials is a complicated process and involves the coordinated activities of many different enzymes and structural proteins, as well as cytoskeleton, at different locations in the cell. In the following, the synthesis and deposition of noncellulosic polysaccharides are presented first, followed by that of cellulose and wall proteins.

3.4.1. Noncellulosic Polysaccharides

The donor of a sugar unit to the growing chain of pectins or hemicelluloses is almost always a nucle-

otide sugar, uridine diphosphoglucose (UDP-Glc), UDP-Gal, UDP-Xyl, or GDP-Fuc, etc. These substrates are believed to be taken up into the lumen of endoplasmic reticulum (ER) or Golgi, where the appropriate enzymes, synthases, and transferases transfer the sugar moiety to the growing oligo- or polysaccharide chain. Each different linkage in a complex oligosaccharide usually requires a separate, specific glycosyl transferase, not modified versions of the same glycosyl transferase. These polysaccharides are synthesized in the lumen of ER and Golgi bodies, modified in various ways, packaged into vesicles, and transported to the cell exterior by a process similar to exocytosis (see Box 2-2).

BOX 2-2 USE OF IMMUNOCYTOCHEMISTRY IN ELUCIDATION OF SYNTHESIS OF WALL POLYSACCHARIDES

THE USE OF POLYCLONAL or monoclonal antibodies against specific fragments (epitopes) of wall polysaccharides provides valuable information on the location of these polysaccharides in the wall, as well as on their cellular sites of biosynthesis and transport. To produce these antibodies, wall polysaccharides are first obtained in as pure a form as possible. Specific endoglycanases cleave the polysaccharide into fragments. These fragments, either by themselves or coupled to proteins, such as bovine serum albumin (BSA), are used to produce antisera, which are fractionated and purified. Polyclonal or monoclonal antibodies are produced as described in Chapter 5.

Materials to be tested, such as cells in suspension culture or whole sections of root or stem, are quick frozen under pressure in liquid N₂ or helium to provide a maximum cooling rate. The frozen tissue is chemically fixed at -70 to -80°C by a fixative, such as osmium tetroxide in acetone, and the ice is substituted at the same time by the acetone. When the substitution is complete, the tissue is gradually warmed to room temperature, rinsed with acetone, and embedded in a plastic, such as Epon (for routine examination) or LR White (for antibody work) for sectioning at <0.1 µm for transmission electron microscopy (TEM).

For immunocytochemistry, sections are stained with a solution of the antiserum in buffer and then double stained with colloidal gold, which is linked to an antibody-binding protein or to a goat or sheep antibody raised against the original antibody. To reduce nonspecific binding of the antiserum, sections are usually immersed in a solution that contains a foreign protein in abundance, such as nonfat milk, before staining with antibodies. Colloidal gold provides the electron contrast needed for visualization of the binding sites under the TEM.

Many antibodies that recognize specific epitopes in polysaccharides have been prepared. Some are shown in Fig. 2-15.

Andrew Staehelin and colleagues at the University of Colorado used these antibodies to localize the synthesis of XGs and pectins in suspension cultured cells of sycamore maple (*Acer pseudoplatanus*). The immunogold-labeling pattern of the anti-XG antibodies showed clearly that XGs are synthesized in Golgi bodies, specifically in the *trans*-Golgi cisternae and the *trans*-Golgi network (TGN) (Fig. 2-16).

The methylation and assembly of pectic polysaccharides, PGA and RG I, also occur in the Golgi bodies, although it is initiated earlier, in the *cis* as well as medial-cisternae. Both XGs and pectic polysaccharides are synthesized in the same Golgi stacks and are translocated via Golgi vesicles to the cell wall.

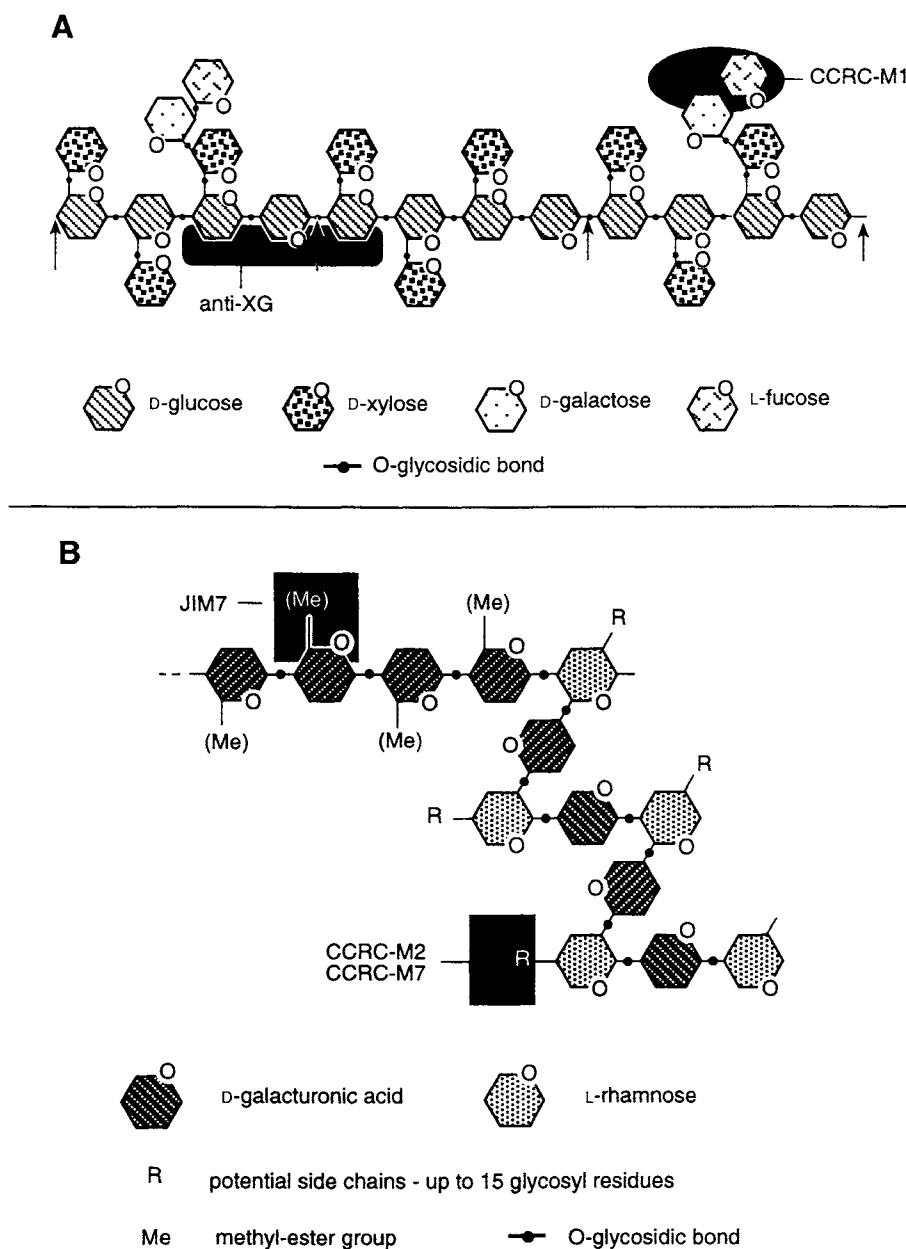


FIGURE 2-15 Parts of polysaccharides showing some of the epitopes and the specific antibodies that recognize them. The polyclonal anti-XG recognizes nearly exclusively the β -1,4-linked glucose backbone of XG; it also binds to solubilized cellulose molecules, but not cellulose fibrils. The monoclonal CCRC-M1 recognizes the terminal fucosyl residue of the trisaccharide side chain of the XG. The monoclonal JIM7 antibody recognizes methyl-esterified GalA units in PGA, but not deesterified GalA. CCRC-M2 is an RG I-specific antibody, it does not recognize PGA. CCRC-M7 recognizes an arabinosyl-containing epitope on RG I. Arrows indicate cleavage sites for the XG chain. From Zhang and Staehelin (1992) with permission.

3.4.2. Synthesis and Deposition of Cellulose

Whereas noncellulosic polysaccharides are synthesized in the ER and Golgi bodies and transported to the

wall in Golgi vesicles, the cellulose chains are assembled and bonded together as microfibrils *in situ* (*in muro*). Cellulose synthase, the enzyme responsible for adding new glucose residues to an existing cellulose

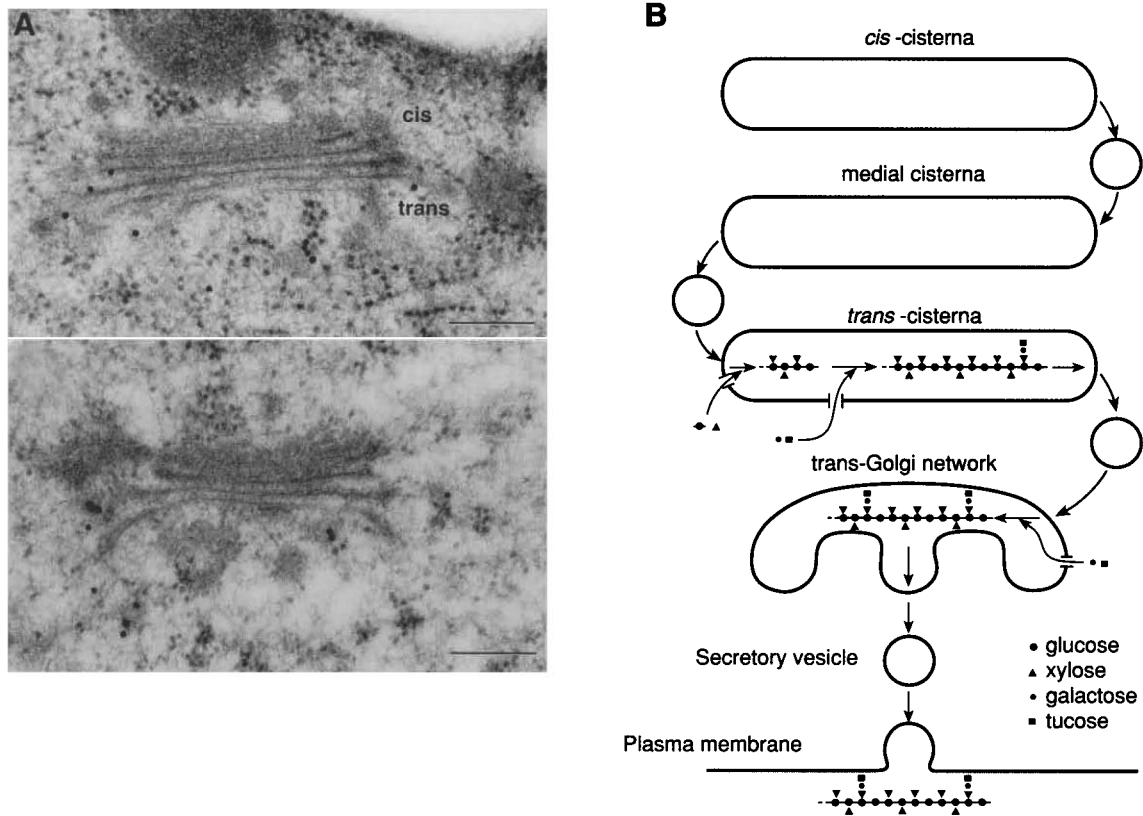


FIGURE 2-16 Synthesis of hemicelluloses in Golgi stacks. (A) Electron micrographs of Golgi stacks of sycamore maple cells immunolabeled with two types of anti-XG antibodies (antibackbone, top; antiterminal fucose on side chain, bottom). All of the label is seen over *trans*-Golgi cisternae and the TGN. Bar: 200nm. Courtesy of Andrew Staehelin, University of Colorado, Boulder, CO. (B) Schematic illustration of synthesis of XGs. Substrate sugars are shown being taken up into the lumen of Golgi cisternae. Secretory vesicles transfer the polysaccharides to the cell wall. From Zhang and Staehelin (1992).

chain, is a transmembrane protein localized in the plasma membrane with substantial parts in the cytoplasm. Arrays of particles on the inner face of the PM purporting to represent glucan synthases have been seen in freeze-fractured and shadowed TEM pictures of PM fractions from higher plants, algae, and bacteria. In high-resolution photographs, these particles (units) appear arranged in rosettes with a hexagonal symmetry, with each unit consisting of six similar subunits (Fig. 2-17).

In the last few years, convincing evidence has emerged that each subunit in the rosette represents a cellulose synthase molecule which adds glucose residues to an existing chain, and that the three-dimensional arrangement of individual synthase molecules in the rosette is responsible for the ordered array of

chains in a microfibril. We start with the intracellular origin of UDP-Glc and its addition to a glucan chain.

In plants, UDP-Glc can be formed in either of two ways:

- i. $\text{Glc1-P} + \text{UTP} \longleftrightarrow \text{UDP-Glc} + \text{PPi}$
- ii. $\text{sucrose} + \text{UDP} \longleftrightarrow \text{UDP-Glc} + \text{fructose}$

The second reaction catalyzed by sucrose synthase (SuSy) is favored because SuSy is found in high levels in nonphotosynthetic organs and tissues. In cotton fibers, which synthesize cellulose in large amounts, a sucrose synthase is found associated with the plasma membrane and could feed UDP-Glc to the cellulose synthase complex. The glucose units were once thought to be added to the reducing end of a β -1,

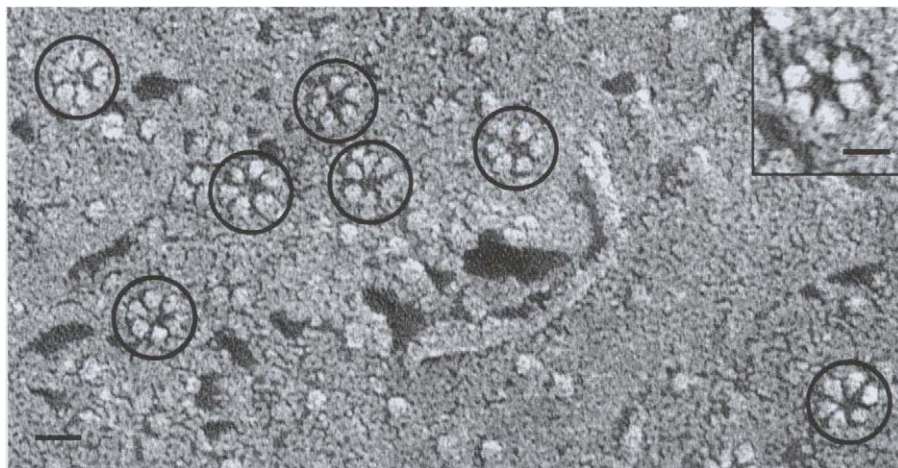


FIGURE 2-17 Arrays of particles on the protoplasmic face of plasmalemma. Particles lie singly, in clusters of two to three particles, or in well-ordered rosettes of six closely associated particles. Seven rosettes are shown (circled). The one rosette in the inset shows the six individual subunits more clearly. To obtain this electron micrograph, the tissue was quick-frozen and fractured under vacuum in a freeze-etch machine. Partial sublimation of water from the tissue (etching) was allowed to occur, and the cell surface was shadow cast with platinum/carbon while the specimen was rotating (rotary shadowing) and coated with pure carbon to provide contrast, relief, and necessary stability under the electron beam. The freeze-fracture technique separates the bilayer of membranes into individual leaflets; in the case of plasmalemma, the extraplasmic face toward the cell exterior is separated from the protoplasmic face (see Fig. 2-20B). The leaflet is shown facing the protoplasm. Plant material is from differentiating xylem cells from *Zinnia* mesophyll. Xylem cells deposit a thick secondary wall rich in cellulose. Magnification bars: main micrograph, 20 nm; and inset, 10 nm. Both micrographs are courtesy of Mark Grimson and Candace Haigler, Department of Biological Sciences, Texas Tech University, Lubbock, TX.

4-glucan chain, but data indicate that they are added to the nonreducing end of the chain, as in other polyglucans, such as starch and glycogen.

3.4.2.1. Cellulose Synthase genes

Some strains of bacteria (e.g., *Acetobacter xylinum*, *Agrobacterium tumefaciens*) synthesize cellulose, and genes encoding cellulose synthases have been cloned from these organisms. However, probes utilizing the *A.xylinum* gene to screen cDNA libraries from plants in a search for homologous genes were unsuccessful because plant genes have many other intervening sequences that have no counterpart in the bacterial genes. Deborah Delmer and associates at the University of California and Calgene, both in Davis, California, using information on conserved sequences from bacterial genes and a number of other glycosyl transferases that utilize UDP-Glc as a donor (e.g., chitinases, hyaluronases), identified two clones in a cDNA library derived from cotton fibers that showed expected sequences. The cDNA clones led to the isolation of genes, now known as *GhCeSA-1* (for gossypium hirsutum cellulose synthase A-locus 1) and *GhCeSA-2* (Pear *et al.* 1996). Since then, several other putative cellulose synthase (*CeSA*) genes have been cloned from *Arabidopsis* and rice (*Oryza sativa*), and the numbers are increas-

ing. The encoded proteins from plants share some conserved domains with the bacterial cellulose synthases; they also show some domains that occur only in plants, not in bacteria (Fig. 2-18). There are also some plant genes, which, like the bacterial genes, lack the plant-specific conserved or hypervariable regions; their roles are still uncertain and are tentatively named cellulose-synthase-like genes.

3.4.2.2. Rosettes Are Important for Formation of Crystalline Cellulose

Several mutants have been identified in *Arabidopsis*, which are defective in cellulose synthesis. *rsw1-rsw3* (for radial swelling) mutants are conditional mutants that show swelling of root tips when grown at high temperature (at low temperature the plants show a normal phenotype), and under severe conditions the seedlings die.

At high temperature, the mutant plants produce a glucan, thought to be a β -1,4-glucan, but they do not produce crystalline cellulose or microfibrils. Plasma membrane preparations from *rsw* mutants show a lack of rosettes with sixfold symmetry. In some *irx* (for irregular xylem) mutants, cellulose synthesis is curtailed, which leads to insufficient deposition of the secondary wall in differentiating xylem vessel

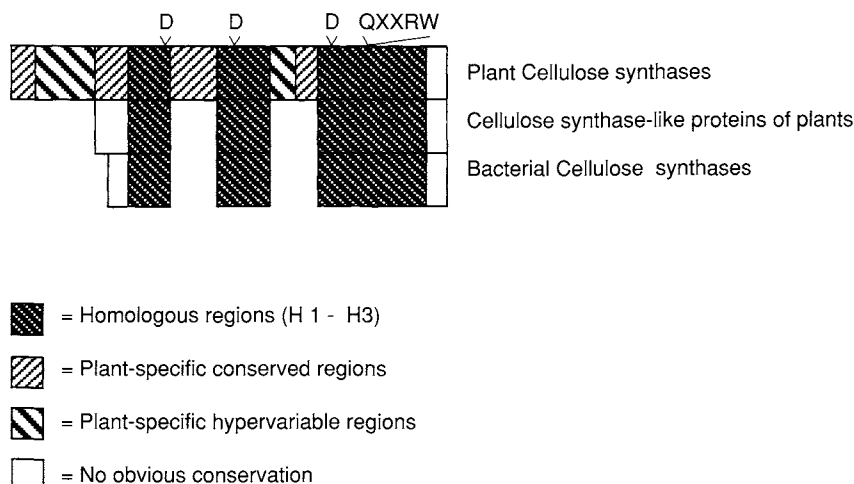


FIGURE 2-18 Domain structure of putative cellulose synthase proteins deduced from *CeSA* genes of plants and bacteria. The proteins share three homologous regions (H1-H3) with good conservation among plants and bacteria. These regions contain the conserved aspartate (D) residues as well as the motif Gln-X-X-Arg-Trp (QXXRW). Intervening domains in plant proteins include some regions that are highly conserved, whereas others are highly variable. From Delmer (1999).

elements and their eventual collapse. The wild-type genes, *RSW1* and *IRX3*, have been cloned and encode proteins similar to bacterial cellulose synthases and even more similar to the *CeSA* genes of cotton. These data suggest that *CeSA* genes are critical for cellulose synthesis and that the three-dimensional arrangement of cellulose synthase molecules is important for the aggregation of individual β -1,4-glucan chains to form a microfibril.

3.4.2.3. Model for Cellulose and Fibril Biogenesis

The deduced structure of plant cellulose synthase proteins indicates the presence of eight transmembrane (TM) domains, and the amino and carboxy-terminals, as well as substantial portions between the second and the third TM domain to reside on the cytoplasmic side of the PM (Fig. 2-19). The eight TM domains are thought to form amphipathic α helices and are so placed in the PM as to form a central pore (or channel) with hydrophobic side chains of amino acids facing the lipid bilayer and polar side chains facing the interior or lining the pore. The intracellular portions carry conserved domains for substrate binding and for catalysis, which are shared with bacterial cellulose synthases. They also carry several conserved plant-specific domains. The plant-specific domains are thought to be involved in functions unique to plants, i.e., binding of sucrose synthase, interaction with proteins associated with the cytoskeleton (see later), or other accessory proteins. Domains that are plant specific, but highly variable, are thought to provide specificity to individual cells/tissues/organs. As shown in later chapters, many key

enzymes occur in multiple isoforms, which show cell- and development-specific expression. Some *CeSAs* have already been shown to display tissue-specific expression.

According to the model, sucrose synthase acts in concert with the cellulose synthase complex. Sucrose synthase supplies UDP-Glc to the substrate-binding domain. The catalytic site cleaves the glucose residue and adds it to the nonreducing end of an existing glucan chain, which is extruded out of the pore formed by the transmembrane domains. Similar models are known for various proton and/or calcium-ATPases, which use energy from ATP hydrolysis to carry protons or calcium ions across cell membranes. One *CeSA* subunit, as shown here, probably interacts with five other such subunits to form one of the six units of the synthase complex, the rosette. Using immunogold labeling and antibodies against the conserved catalytic site of the cotton cellulose synthase, it has been shown that the antibodies indeed bind to the rosettes in the freeze-fractured plasma membrane of another dicot, *Vigna angularis*. Such recognition provides the first direct evidence that cellulose synthase is a component of the rosettes in higher plants.

While some features of cellulose chain elongation and fibril biogenesis have been clarified, some other questions remain. It is unknown how the different cellulose synthase molecules interact to form the complex. It is also unclear how every alternating residue in the chain is rotated by 180° such that intra- and inter-chain bonds are formed to give rise to a cellulose fibril.

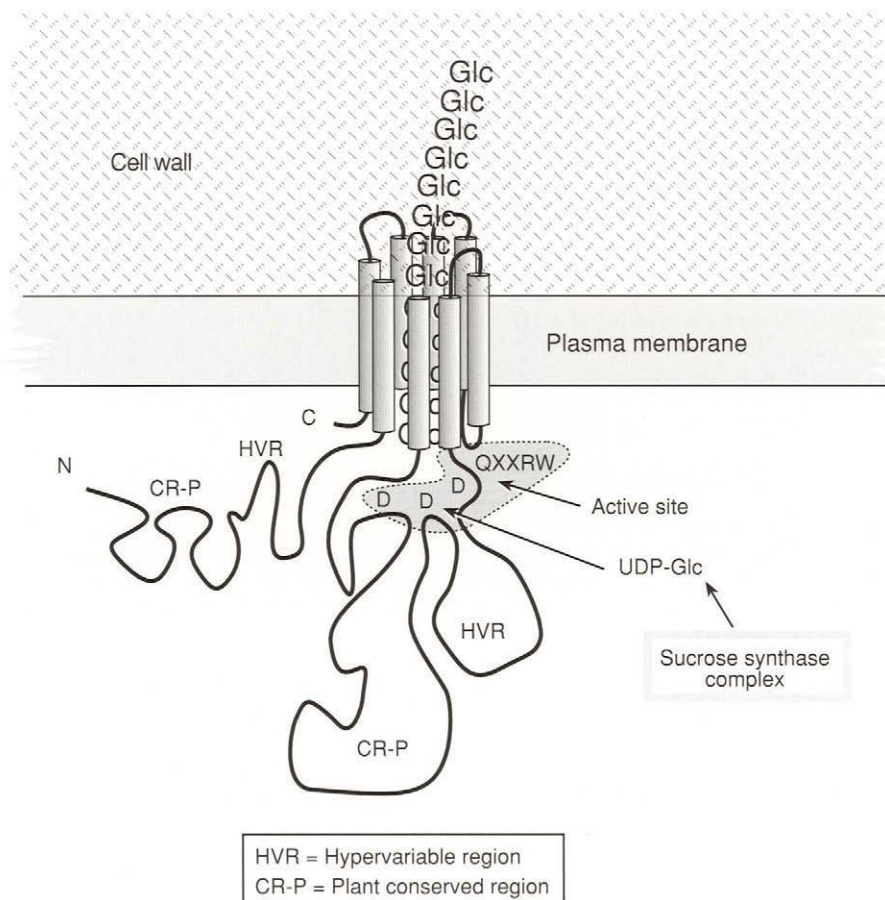


FIGURE 2-19 Hypothetical model for the topology of one cellulose synthase (CeSA) protein subunit in the PM of plants. The eight transmembrane helices are predicted to form a pore through which the cellulose chain (Glc-Glc-Glc) is secreted to the cell wall. The large central domain is thought to fold in such a way as to bring together the conserved regions containing the three aspartate (D) residues and the Gln-X-X-Arg-Trp (QXXRW) motif, which are believed to be important for substrate binding and catalysis. This would place the conserved and hypervariable plant-specific regions also in the cytoplasm, where they may serve to interact with other proteins. From Delmer (1999).

Several possibilities have been suggested. For instance, two synthases in opposite configuration could add glucose units from UDP-Glc synchronously on the same chain. Alternatively, cellobiose units, not glucose units from UDP-Glc, may be the building blocks. However, for various reasons, these possibilities are unsatisfactory. It is also unclear whether the particles that occur singly or in groups of two or three on the protoplasmic face of the plasmalemma are artifacts of preparation, whether they synthesize smaller diameter cellulose fibrils than the rosettes, or whether they are involved in the synthesis of polymers other than cellulose (e.g., callose).

3.4.2.4. Regulation of Cellulose Fibril Orientation

It was mentioned earlier that cellulose fibrils within a layer are oriented predominantly in one direction. How is such uniform arrangement achieved? Abun-

dant evidence shows that microtubules in the cell cortex, called cortical MTs, play a role in the orientation of new cellulose fibrils. This is supported by a parallelism between the orientation of cellulose fibrils adjacent to the plasmalemma and the orientation of the cortical MTs immediately subjacent (Fig. 2-20A). How the cortical MTs control the orientation of cellulose fibrils is not known, but it seems that the MTs and plasmalemma form a complex, functional unit that regulates the orientation of cellulose chain assembly and thereby the orientation of cellulose fibrils. Cortical MTs are believed to provide a fretwork against the plasmalemma, which directs and regulates the flow of Golgi vesicles, as well as cellulosic precursors toward the plasmalemma. A model showing the complex interaction among cortical MTs, plasmalemma, arrays of cellulose synthase units, and cellulose fibrils is shown in Fig. 2-20B. Actin fibrils are

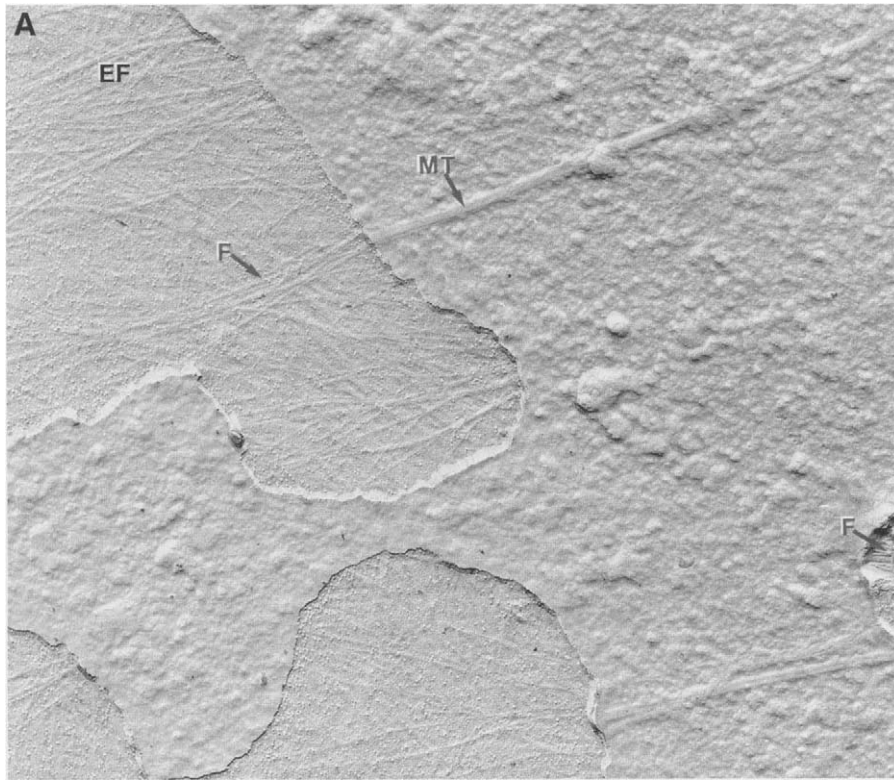


FIGURE 2-20 (A) Electron micrograph showing the plasmalemma, microtubules, and cellulose fibrils. The freeze-fracture face is viewed as if from the inside of a cell looking out toward the wall. This is an exceptionally fortunate photograph because the fracture passes at different levels across the cell surface, revealing the inner leaflet of plasmalemma, and also parts where it has broken off, revealing the extraplastic face (EF) of the outer leaflet. The cytoplasmic side of the inner leaflet of plasmalemma shows impressions of cortical microtubules, and the EF of the outer leaflet shows an impression of the most recently deposited cellulose fibrils (F). Note the parallelism between the orientations of MTs and cellulose fibrils. X 22,000. (B) A model showing relationship among MTs, plasmalemma, particles believed to be cellulose synthase, and cellulose fibrils. This model also shows how the bilayer of plasmalemma in freeze-fracture preparations, is split in the middle, exposing the protoplasmic face (PF) and the extraplastic face (EF). From Gunning and Steer (1996).

not shown in this model, but they are also involved in the process, probably by maintaining the framework of cortical MTs in place. Drugs that depolymerize either actin (e.g., cytochalasin B) or MTs (e.g., colchicine) affect the pattern of newly deposited cellulose fibrils.

3.4.3. Deposition of Proteins

The various structural proteins are synthesized on the ER, processed and glycosylated within the ER and Golgi bodies, and translocated to the wall via exocytosing vesicles. Extensin monomers are synthesized and secreted through the plasmalemma as a water-soluble precursor protein. Once in the wall, they are linked into an insoluble extensin network (for details of protein processing and targeting, see Appendix 3, in book Section III).

3.5. Dynamism in Wall Structure

Primary cell walls are highly dynamic structures that show constant changes during cell growth, cell division, pathogen attack, wounding, etc. Cellulose fibrils, once deposited, do not show much turnover. This has been confirmed by proton magnetic resonance spectroscopy, although they may and do change their orientations with cell growth. In contrast, matrix polysaccharides, pectins, and hemicelluloses, including xyloglucans and xylans, show considerable turnover. This is confirmed readily by analysis of culture or incubation media, especially if wall polysaccharides have been previously radiolabeled. For instance, oligosaccharide fragments from xyloglucans are released into the incubation medium during auxin-induced growth. Many other oligosaccharides and wall fractions are released during wounding and/or pathogen

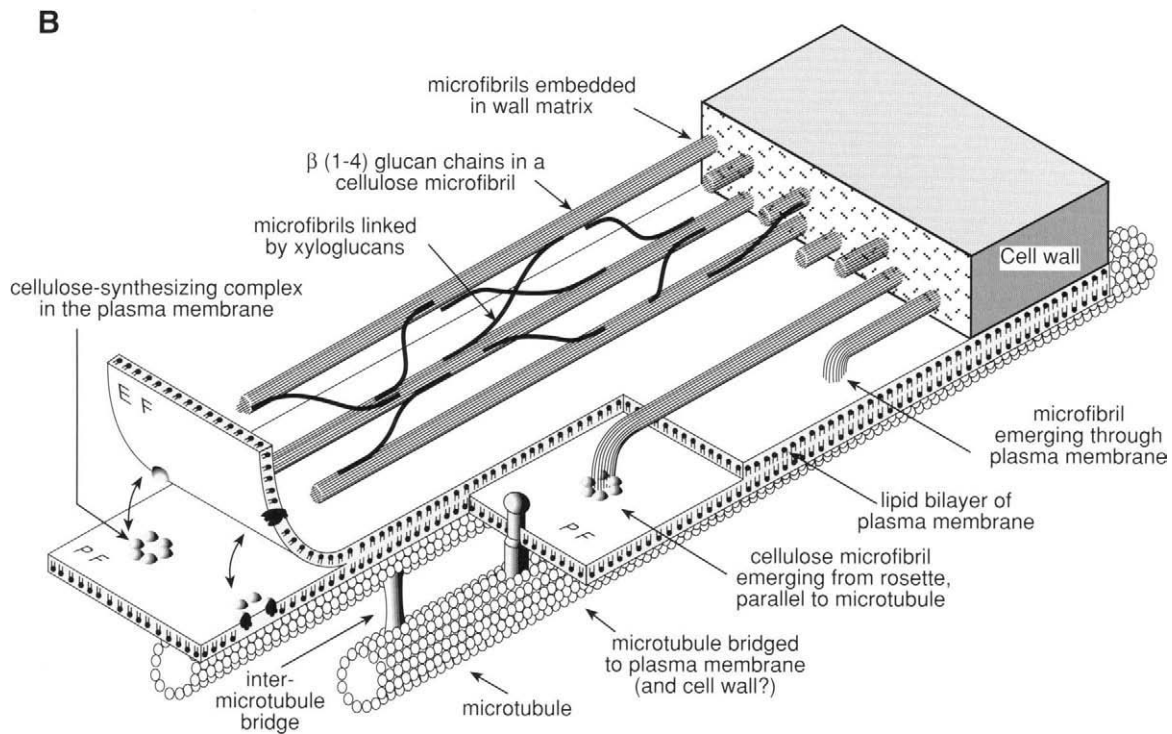


FIGURE 2-20 (continued)

attack and, in some instances, have been shown to elicit defense responses from the plant (see Chapter 12, Box 12-1). It should also be remembered that during the growth of cells, new cell wall materials, including cellulose and matrix polysaccharides, are synthesized and deposited in the growing wall. This deposition requires adjustments in arrangement of the existing materials (see Chapter 15; also Section III.2 in this chapter).

4. WALLS OF SPECIALIZED CELLS

4.1. Epidermal Cells

Epidermal cells in aerial parts of plants have middle lamella and primary walls as discussed earlier, but they also show some special features. The outer tangential walls of epidermal cells usually have a multi-layered structure in which the orientation of cellulose fibrils alternates from being more or less horizontal to being longitudinal. In addition, they deposit a layer of cuticle on their outer surface. Cuticle is a layer of cutin mixed with waxes that is deposited as a more or less homogeneous layer (Fig. 2-21A). It is separated from the cellulosic part of the primary wall by a pectic layer (Fig. 2-21B) and can be easily peeled off as a

layer by treatment with solutions of pectinase or ammonium oxalate. Cutin is a polymer of C_{16} and C_{18} fatty acids, with one or more hydroxy groups or epoxides, held together mainly by primary alcohol ester linkages. The monomers are secreted out of the epidermal cells and polymerize on the external surface. Some other cell types, such as endodermis in roots and cork cells in periderm, also contain lipids.

4.2. Collenchyma

Collenchyma tissue consists of specialized parenchyma cells that develop unevenly thickened primary walls (Fig. 2-22). The thickenings are usually at the corners, although they may also occur along longitudinal walls. The thickenings are particularly rich in pectic polysaccharides, which occur interspersed between lamellae of cellulose fibrils. Collenchyma tissue is adapted for mechanical support, but at the same time it is able to stretch. Thus, it is designed to provide support to aerial organs, such as herbaceous stems and petioles, that are still elongating.

4.3. Lignified Cells

Lignin is a phenolic compound deposited in the walls of certain cell types that have stopped enlarging or

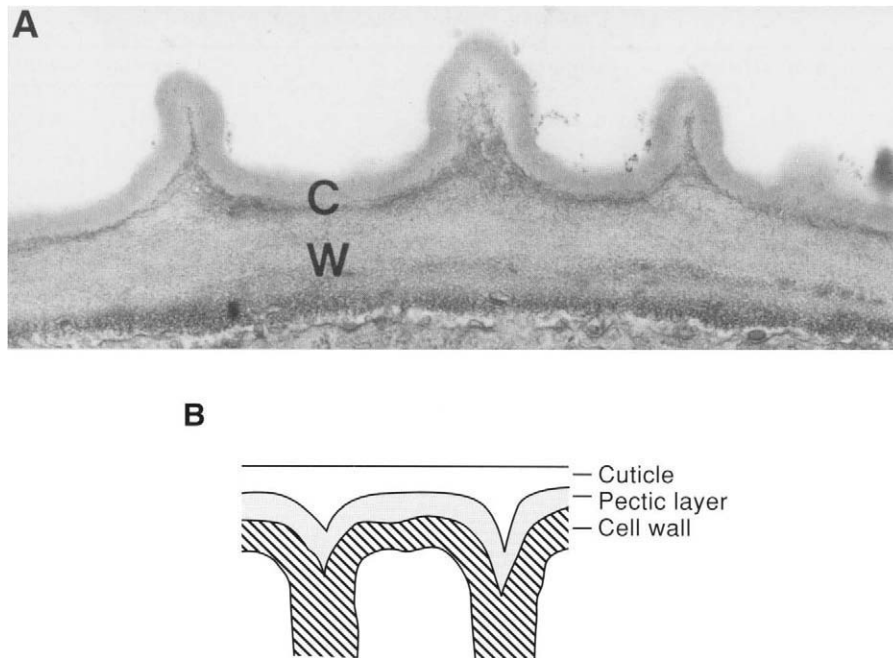


FIGURE 2-21 (A) Cuticle on epidermis of daffodil flower corona. In this epidermis, cuticular ridges are seen. C, cuticle; W, cell wall. $\times 24,000$. From Gunning and Steer (1996). (B) Diagrammatic representation of the relationship between cuticle and epidermal cell wall. From Kolattukudy (1984).

elongating and mostly in cells involved in the conduction of water and minerals and/or mechanical support. Prime examples are tracheids and vessel elements (jointly referred to as tracheary cells or elements) in xylem tissue (wood is secondary xylem), fibers, scler-

eids, and lignified parenchyma. Sclerenchyma is a general term used to denote a tissue or cell type that has lignified walls.

Plants produce hundreds of different types of phenolic compounds (see Table 2-3).

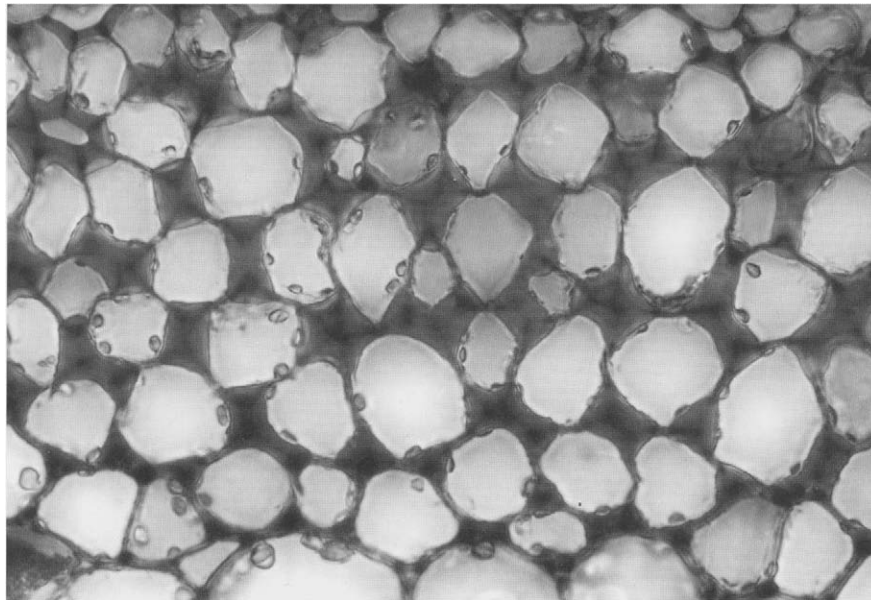
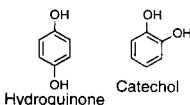
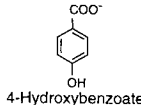
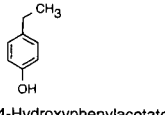
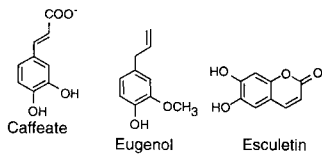
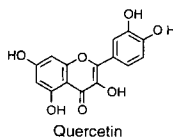
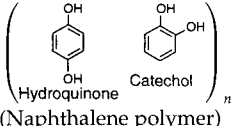
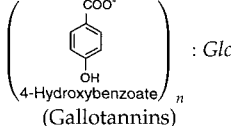
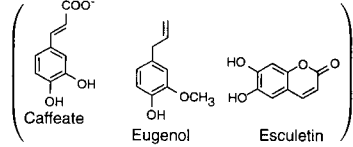
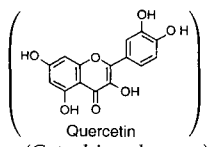


FIGURE 2-22 Part of outer cortex of bean (*Phaseolus vulgaris*) stem showing collenchyma cells in cross section. Cell wall materials rich in pectins are deposited primarily at cell corners. These cells provide mechanical support in organs that are still growing.

TABLE 2-3 Common Phenolic Substances in Plants^a

No. of Carbon atoms	Carbon skeleton	Compound class	Compound example
<6>	C ₆	Simple phenols	 Hydroquinone Catechol
<7>	C ₆ -C ₁	Hydroxybenzoates	 4-Hydroxybenzoate
<8>	C ₆ -C ₂	Phenylacetates	 4-Hydroxyphenylacetate
<9>	C ₆ -C ₃	Hydroxycinnamates Phenylpropenes Coumarins	 Caffeate Eugenol Esculetin
<15>	C ₆ -C ₃ -C ₆	Flavonoids	 Quercetin
<n>	(C ₆) _n	Catechol melanins	 (Naphthalene polymer)
	(C ₆ -C ₁) _n : Glc	Hydrolyzable tannins	 (Gallotannins)
	(C ₆ -C ₃) _n	Lignins	 (Guaiacyl lignins, Guaiacyl-syringyl lignins)
	(C ₆ -C ₃ -C ₆) _n	Condensed tannins	 (Catechinpolymers)

^aAdapted from Strack (1997).

Lignin is a polymer of phenyl propanoid (C_6-C_3) units. Three such subunits, *p*-coumaryl, guaiacyl, and sinapyl propane subunits, derived from hydroxycinnamic alcohols, *p*-coumaryl, coniferyl, and sinapyl alcohols, respectively, are linked by a variety of bonds to give rise to a highly complex and branched polymer (Figs. 2-23A and 2-23B). Lignins from different species, and from different tissues in the same plant, show different proportions of the three types of building units. For instance, gymnosperm woods have a high proportion of guaiacyl units, whereas those from dicots have about equal amounts of guaiacyl and sinapyl units; sclerenchyma tissues from some monocots have all three. The monomers are glucosylated prior to being secreted into the wall, where they are deglucosylated before polymerization. The initial linkage may be provided by ferulic acid, which is linked to pectins, but a further polymerization pattern is highly complex and irregular because it is believed to be nonenzymatic.

Polymerization apparently continues until precursors and/or space is used up. The result is a highly hydrophobic, strong, meshwork that surrounds other

wall components and cements them in place (Fig. 2-23C). Plant tissues with lignin in their cell walls are tough, rigid, strong, but still elastic properties that make wood such an ideal material for construction and furniture. Because lignin is hydrophobic, it is also impermeable to fluids and is an effective barrier to pathogens.

Once deposited, lignin cannot be removed from the cell wall because plant cells lack ligninases, or lignin-digesting enzymes. Several bacteria and fungi have lignin-digesting enzymes and thus serve in the natural decay of wood.

4.4. Suberized Cells

Cork cells in periderm, exodermis in roots, Casparian strips in endodermis, and also many other cell types deposit suberin. Suberin is deposited in layers outside the plasma membrane, but within the primary wall (Fig. 2-24A). The layers also contain wax. Suberin contains aliphatic components, as well as aromatic components of the lignin type. The aliphatic components are

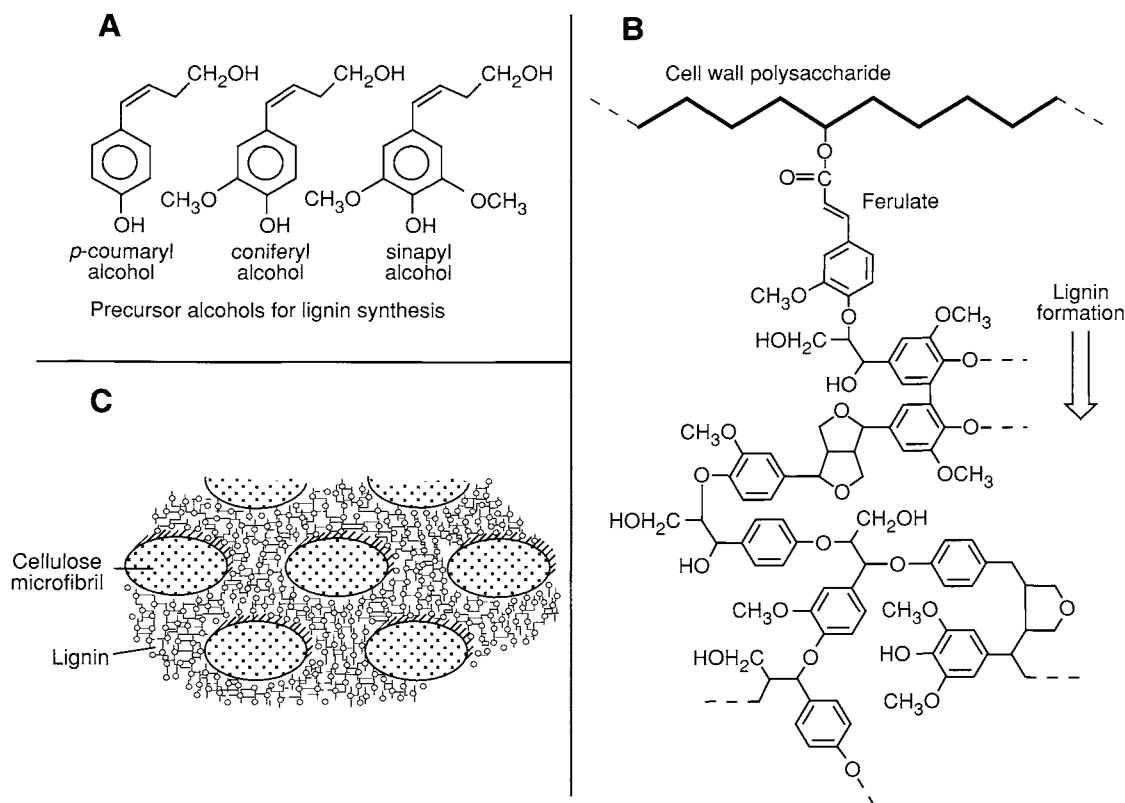


FIGURE 2-23 (A) Precursor alcohols of lignin biosynthesis, *p*-coumaryl, coniferyl, and sinapyl alcohols. From Strack (1997). (B) Postulated initial steps in lignification. Only a small part of the lignin molecule is shown. From Strack (1997). (C) Schematic drawing showing relationship of lignin and wall polysaccharides. Only cellulose fibrils are shown. From Brett and Waldron (1996).

characterized by the presence of very long chain hydroxy (C_{20} to C_{32}) fatty acids and fatty alcohols, which are rare in cutin, joined together by ester links (Fig. 2-24B). The aromatic components are poorly known but, on complete digestion, give aldehyde precursors of lignin monomers—*p*-hydroxybenzaldehyde, vanillin plus small amounts of syringaldehyde. They are probably linked as in lignin. It is believed that aromatic components are bound to the cell wall and that aliphatic components are anchored to the phenolic components.

5. SECTION SUMMARY

Cell walls are complex structures designed to serve many important roles (e.g., support, protection, uptake of substances). All cells in multicellular plants have middle lamella and primary walls. Some cells also deposit secondary walls after their growth has ceased. Because all cells that are dividing or growing have to contend with middle lamella and primary wall, these wall layers are the main focus of this chapter. Different types of polysaccharides, proteins, and phenolics provide the structural brick and mortar for the walls, although their proportions vary in different wall layers and in different taxa. Some special cell types also deposit lipids. Among the polysaccharides, cellulose is composed of long, straight chains of glucose residues. Several such chains come together to form a cellulose microfibril. Microfibrils are tethered together via short chains of hemicelluloses and provide the structural framework for the wall. Other hemicelluloses and pectins provide the matrix material; pectins specially serve other roles in ionic balance, pH regulation, porosity, and permeability. Several glycoproteins rich in hydroxyproline, or proline or glycine, are also present, although their exact roles are uncertain. Enzymes involved in growth or wall modifications, in plant defense, and in other functions also occur in and are associated with cell walls. All noncellulosic polysaccharides are synthesized from precursors in the ER and Golgi bodies and are transported to the wall *via* Golgi vesicles. Remarkable progress has been achieved in our understanding of cellulose synthesis in plants since the mid-1990s. The cloning of the first plant cellulose synthase gene has led to detailed investigations on the occurrence and structure of *CeSA* genes in plants. Cellulose synthase is a complex molecule, which is predicted to have transmembrane domains that form a pore in the plasmalemma and cytosolic domains involved in substrate recognition and catalysis, as well as inter-

actions with the enzyme complex that donates glucose and with cytoskeletal proteins. Cellulose synthase molecules are arranged in the plasma membrane in a hexagonal symmetry, called rosettes. The newly added β -1, 4-linked glucan residues are extruded out in chains through the pore, and the arrangement in rosettes ensures that the extruded chains are zippered up to give rise to the finished product, the microfibril. There are indications that families of *CeSA* genes exist in a single plant (e.g., maize, *Ara-bidopsis*) and that different members are expressed to varying levels in different tissues and wall layers. Brief descriptions of walls in epidermis, collenchyma, lignified cells, and suberized cells are given.

SECTION II. CELL DIVISION

1. CELL CYCLE

The cell cycle in eukaryotes typically has four phases. These phases include the synthetic or S phase, when DNA is duplicated and associated proteins such as histones are synthesized, and the mitotic or the M phase during which mitosis occurs, quickly followed by cytokinesis. Alternating with the S and M phases are two interphase periods: the Gap1 or G_1 phase and the Gap 2 or G_2 phase (see Fig. 2-25). G_1 and G_2 are not periods of “rest” as the designation “interphase” might suggest. Indeed, they are periods during which somatic and vegetative cells must grow prior to further cell cycle progress and factors controlling cell differentiation and cell division operate.

The duration of individual phases in the cycle varies considerably, though generally the S and the M phases are much shorter than the G_1 or G_2 . For instance, in root tip cells of wheat, the entire cycle may take 18–24 h, with the S phase occupying 3–4 h and the M phase 2–3 h. In rapidly cycling cells as of an embryo, however, G_1 and G_2 may be very short. The duration of the whole cycle also varies widely among tissues and organs of the same organism (see Fig. 1-5B, Chapter 1, which shows that cells in peripheral positions in the root apex cycle much faster than those in the quiescent center). When plant cells stop dividing, they are arrested in either G_1 or G_2 . Because cells in G_2 have already gone past the S phase, their DNA content will have doubled relative to the G_1 phase and, in nonpolyploid cells, will be 4C, as opposed to 2C in cells arrested in G_1 (1C is the haploid DNA content), and this difference can be measured spectrophotometrically (see Box 15-1, Chapter 15).

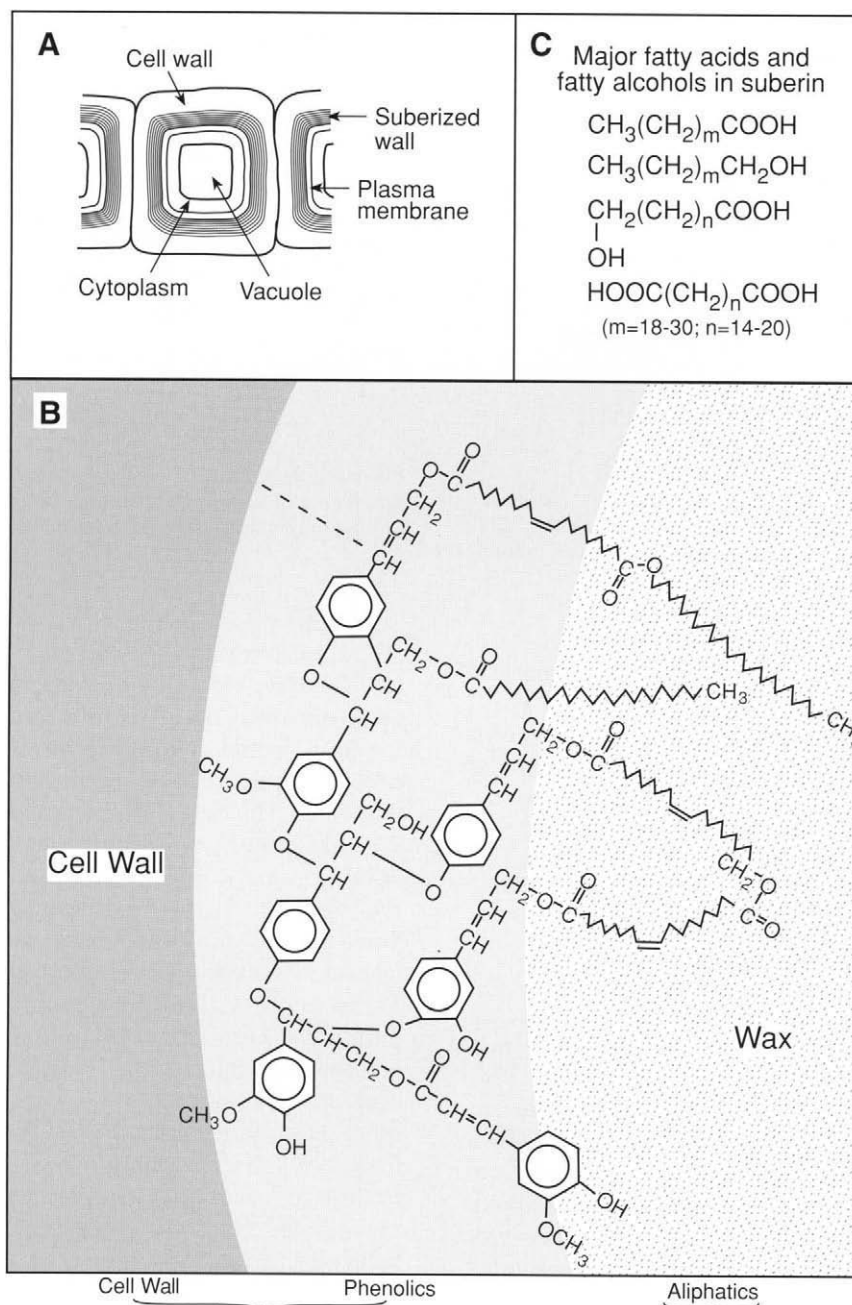


FIGURE 2-24 (A) The location of suberized layers between the plasma membrane and the cell wall. (B) The relationship between the cell wall and the aromatic and aliphatic components of suberin. (C) Major components of subenn. From Kolattukudy (1984).

1.1. Checkpoints

For orderly development and differentiation of an organism, it is essential that cell divisions be precisely controlled. One level at which such control is exercised is at the level of cell cycle, i.e., the entry of cells into the cycle and their progress through mitosis and cytokinesis. The cell cycle is precisely regulated with strong

checkpoints at the G_1 to S (G_1/S) transition and at the G_2 to M (G_2/M) transition. These checkpoints ensure that conditions are right for cells to engage in another round of duplication of DNA in the S phase or for cells to enter the complicated chain of events that is called mitosis. For example, before entering S phase, it would be of benefit to a cell's progeny, and thereby the organism of which it is a part, to confirm that the last

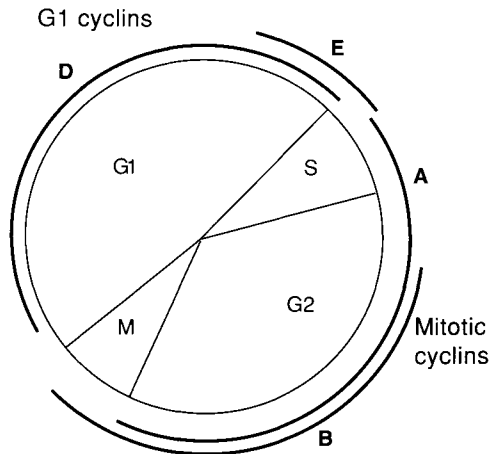


FIGURE 2-25 Cell cycle and relative abundance of different cyclins. Cyclins A and B are mitotic cyclins; D and E are G1 cyclins. Dividing cells generally spend more time in G₁ and G₂ than in S and M phases.

mitosis has been completed; that the cell has enlarged sufficiently since the last cytokinesis; and that the ambient conditions will support a complete genome replication. Likewise, to preserve viability and the integrity of the genome, the cell must not enter M phase from G₂ unless DNA synthesis is complete and damaged DNA is repaired.

2. CYCLINS AND CYCLIN-DEPENDENT KINASES

Regulation of the cell cycle is highly complex. Processes such as DNA synthesis, mitosis, and cytokinesis, each in itself highly complex, are coordinated by a variety of regulatory proteins and enzymes and are carried through in a precise sequence and timely manner. The mechanisms, in general, are highly conserved in all eukaryotes. Among the various proteins involved, the concerted actions of two classes of proteins called **cyclins** and **cyclin-dependent kinases** or **CDKs** play the major role. Cyclins are regulatory proteins without which CDK subunits cannot be enzymatically active; whereas CDKs are kinases, enzymes that phosphorylate other proteins. The two are separate proteins, but they form a dimeric functional unit, the cyclin-CDK complex (Fig. 2-26). The complex exists in multiple forms by combinations of CDK with different cyclins and drives the cell cycle progression through the control points of G₁/S transition or the G₂/M transition. Higher plants and metazoa have several CDKs whereas lower eukaryotes have a single CDK.

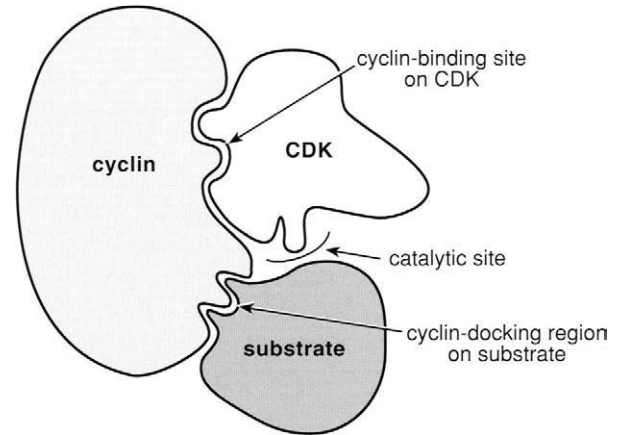


FIGURE 2-26 Schematic representation of a cyclin and a CDK. The two proteins form a functional complex that is essential for driving the cell cycle through the control points.

Cyclins were first identified as proteins that became abundant for a short time before each mitosis in rapidly dividing egg cells of invertebrates. In these eggs, early divisions occur, without growth, by partition of the large initial egg; hence, in the absence of general protein synthesis, the synthesis of cell cycle proteins was clear. The first CDK identified was the product of the *CDC28* gene of budding yeast (*Saccharomyces cerevisiae*), dramatically shown to be homologous with the *cdc2* gene of fission yeast (*Schizosaccharomyces pombe*). Since then, cyclins and CDKs have been isolated and cloned from many other eukaryotes, including plants. They are best characterized in yeast, fruit fly, and mammals (humans), and much of the terminology comes from those organisms. Regulation of the cell cycle in yeast has served as a model for regulation in other eukaryotic organisms. The following sections first look at the structure and types of CDKs and cyclins in yeast and mammals and some important aspects of cell cycle regulation in those organisms before considering plants.

2.1. Cyclin-Dependent Kinases (CDKs)

CDKs are relatively conserved proteins. In the same organism, far fewer CDKs occur than cyclins, and indeed budding and fission yeast have a single CDK. The same CDK may complex with different cyclins for the two different transitions. Thus, in the fission yeast, the same CDK, p34^{cdc2}, henceforth called simply Cdc2, is complexed with different cyclins at the G₁/S or G₂/M transition. In contrast with fission and budding yeast, animal cells have many CDKs. For instance, as many as nine CDKs have been identified in humans, some are enzymatically active at the G₁/M transition,

whereas at mitosis only Cdc2 (CDK1) is active. In plants, more than one CDK is active at mitosis.

CDKs are serine/threonine kinases, i.e., they catalyze the transfer of a phosphate group from ATP to specific serine or threonine residues in substrate proteins. The substrates include regulatory proteins, such as transcription factors, as well as structural proteins, such as those associated with cytoskeleton or with nuclear membrane. CDKs show several conserved regions. In addition to the catalytic domain, they have an ATP-binding domain. Many CDKs also share a common motif, a 16 amino acid sequence (EGVP-STAIRESLLKE), abbreviated as PSTAIRE, which is a region involved in binding to cyclins. The PSTAIRE motif is present in Cdc2 in fission yeast and CDC28 in budding yeast, and probes utilizing this motif have been used to fish out Cdc2 homologues in many organisms. Other CDKs with more specialized and limited roles in the cell cycle have a partial PSTAIRE motif or a variation on the sequence, which is likely involved in cyclin binding.

2.2. Classification and Structure of Cyclins

In contrast to the relative conservatism of CDKs, cyclins are an abundant and diverse group of regulatory proteins, each of which has affinities for particular substrates of CDK. The cyclin subunit determines which proteins are held close to the CDK and can become substrates, whereas the CDK determines where in the substrate phosphorylation will occur. Thus, while CDKs phosphorylate proteins, cyclins determine the choice of substrate proteins, as well as the timing and intracellular location of phosphorylation. The entry of quiescent cells into the cell cycle is highly regulated and modulated by environmental and hormonal factors in a species- and organ-specific manner. Active cell proliferation involves association of CDKs with cyclins, and the multiple facets of cyclin function make them one of the more complex and fascinating proteins for study. In more recent years, evidence has accumulated that cyclins serve not only to determine the choice of substrates for their cognate CDKs, but also participate in DNA replication, mitotic progress, and cytoskeletal deployment during the cell cycle.

In fission yeast, five to six cyclins are known, although the cell cycle can be successfully completed in genetically modified cells able to express only the mitotic cyclin B (encoding *cdc13*). The number of cyclins is larger in the budding yeast, and the total numbers identified from cDNA clones in multicellular organisms, including plants, run into hundreds, although an individual cell may express only a dozen or so. Cyclins are classified on the basis of their amino

acid sequence and the point or period in the cell cycle during which they activate their CDK partners. The classification is based on mammalian cyclins. Two broad classes are recognized: mitotic cyclins and G₁ cyclins (Fig. 2-25). Mitotic cyclins include types A and B. Type A cyclins appear in the S phase, are maximally expressed in G₂, and decline in content by early metaphase. Type B cyclins appear in G₂ and are maximally expressed in late G₂ and M phases and disappear after metaphase, depending on the type of cyclin. G₁ cyclins include types D and E and are maximally expressed in early to late G₁ and disappear by the end of S phase. Among these, D-type cyclins are better known; they are inducible by peptide growth factors and thus are thought to connect an extracellular signal to induction of the cell cycle. In addition to the just-named cyclins, some less characterized mitotic and G₁ cyclins are known from mammals also.

2.2.1. Structure of Cyclins

Cyclins are 30- to 65-kDa proteins, which show considerable sequence heterogeneity commensurate with their multiple roles, but they also share some common structural motifs. Both mitotic and G₁ cyclins are characterized by the presence of an approximately 100 amino acid conserved sequence, known as the cyclin box, where the CDK partner binds (Fig. 2-27). The cyclin box is part of a larger sequence known as the cyclin fold with five α helices. Two such folds occur in each protein, and the cyclin box spans over helix 1 through helix 5 of the first cyclin fold.

Cyclins are transient proteins and show a high turnover during the cell cycle. The mitotic cyclins, A and B, carry an amino acid recognition sequence at their N-terminal, called destruction box, which targets them to the ubiquitination pathway of proteolysis (for this pathway, see Chapter 22). G₁-type cyclins carry a conserved amino acid sequence called PEST, which is rich in proline, glutamine, serine, and threonine residues (see Fig. 2-27). The PEST sequence occurs in proteins with a fast turnover rate and is also believed to target proteins for ubiquitination. Some cyclins have both a destruction box and a PEST sequence.

3. REGULATION OF CELL CYCLE

Regulation of the cell cycle is highly complex. It requires, among others, activation of regulatory proteins that, in turn, activate the transcription of genes for enzymes involved in DNA and histone synthesis, dissolution of nuclear envelope, and synthesis and/or rearrangement of cytoskeletal proteins, e.g., tubulin

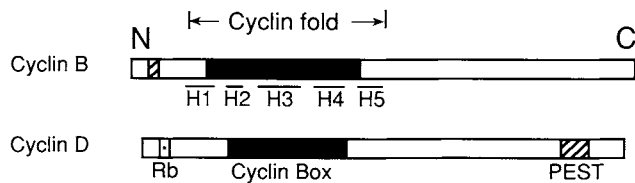


FIGURE 2-27 Schematic representations of structures of a mitotic (B type) and a G1 (D type) cyclin. Approximate locations of the two sets of five α helices (H1–H5) in the first cyclin fold are indicated. The binding site for a CDK, the cyclin box, is shown for both cyclins. Mitotic cyclins have a destruction box (RxxL[x]2–4xxN, where x is a variable amino acid), which occurs near the N-terminal (hatched box). G1 cyclins have the PEST sequence, which occurs near the carboxy terminus. D-type cyclins among them also have a binding suite for retinoblastoma (Rb) protein (see Section 3.3). Modified from Sorrel *et al.* (1999).

and associated proteins. Several processes acting in concert ensure that the cell cycle progresses smoothly. These processes include (i) the sequential activation of specific CDK/cyclin complexes by synthesis/degradation and activation/deactivation of component partners; (ii) phosphorylations that activate or inhibit the participating CDKs; and (iii) the activity of inhibitory proteins.

3.1. Relative Abundance of Cyclins and CDKs

The abundance of mitotic or G₁ cyclins varies considerably at different stages of cell cycle and is regulated at the transcriptional level and by differential proteolysis. For instance, the G₁ cyclins, D and E, are expressed at different times in G₁ and are mostly destroyed by the end of the S phase. The mitotic cyclins, A and B, appear in S or G₂, are stable through prophase, and are rapidly proteolysed during metaphase and anaphase. In contrast, CDKs are regulated primarily at the posttranscriptional level by phosphorylation and by association with cyclin regulatory subunits. For instance, in yeast, *cdc2* mRNA and the steady-state level of *cdc2* protein remain more or less constant with respect to total cellular protein. Similarly, in mammalian cells, the amounts of CDKs in cycling cells are relatively stable. However, this stability is more apparent than real because it is maintained by variations in synthesis and degradation. The pool is almost 75–80% turned over in late G₁ in animal cells.

3.2. Phosphorylations/Dephosphorylations Play a Central Role in Cell Cycle Regulation

The phosphorylation status of cellular proteins is especially high in proliferating cells, particularly at

mitosis, and several different kinases, as well as phosphatases, participate in various reactions at different stages. To give an illustration, the G₂/M transition depends on the activity of one of the most highly conserved complexes: between a cyclin B and a *cdc2* kinase. This complex requires phosphorylation and dephosphorylation at specific amino acid residues. In higher eukaryotes, the complex is catalytically energized by an activating phosphorylation at Thr-161 (in yeast at Thr-167), but is kept deactivated by an inhibitory phosphorylation at Tyr-15 (in some CDKs also Thr-14), which is thought to prevent ATP binding. At the appropriate signal, a specific phosphatase, Cdc25, removes the phosphate group from Tyr-15 (and Thr-14), while Thr-161 (or Thr-167) remains phosphorylated (Fig. 2-28A). As a result, the complex is fully active and cells are driven into mitosis. Homologues of Cdc25 phosphatase are known from a range of species.

3.3. Other Regulatory Proteins

Many proteins are involved in modulating the entry of cells into cell cycle. In mammalian systems, cell divisions are promoted by various chemicals, including peptide growth factors. These substances, known as mitogens (agents that promote mitosis), signal quiescent cells to enter the cell cycle. A special phosphorylation/dephosphorylation cascade, known as mitogen-activated protein kinase (MAPK) cascade, mediates the transmission of the external signal to the nucleus, leading to the synthesis of cyclins specific to the G₁/S transition (for more on the MAPK cascade, see Chapter 25). The retinoblastoma protein (Rb) needs specific phosphorylation by a D-type cyclin–CDK complex to release the transcription factors that in turn activate genes for DNA replication (see Fig. 2-28B). Not only are specific signals required to initiate the entry into the cell cycle, specific proteins also stop vertebrate cells from entering the cell cycle. Thus, small molecular weight proteins known as cyclin kinase inhibitors (CKIs) specifically target the cyclin–CDK complex in G₁ and block the passage of the cell to S phase and a new round of division (see Fig. 2-28B). Still other proteins may act in a cell-/tissue-specific manner and/or in a developmental context to modulate the catalytic activity and/or substrate specificity of the cyclin–CDK complex.

In summary, progression through the cycle depends on the sequential activation and deactivation of distinct CDK–cyclin complexes, which in turn is controlled by stage-specific synthesis/destruction and activation/deactivation of the component subunits. It is also regulated by activating or inhibitory phosphorylations of CDKs as well as the activity of inhibitory proteins.

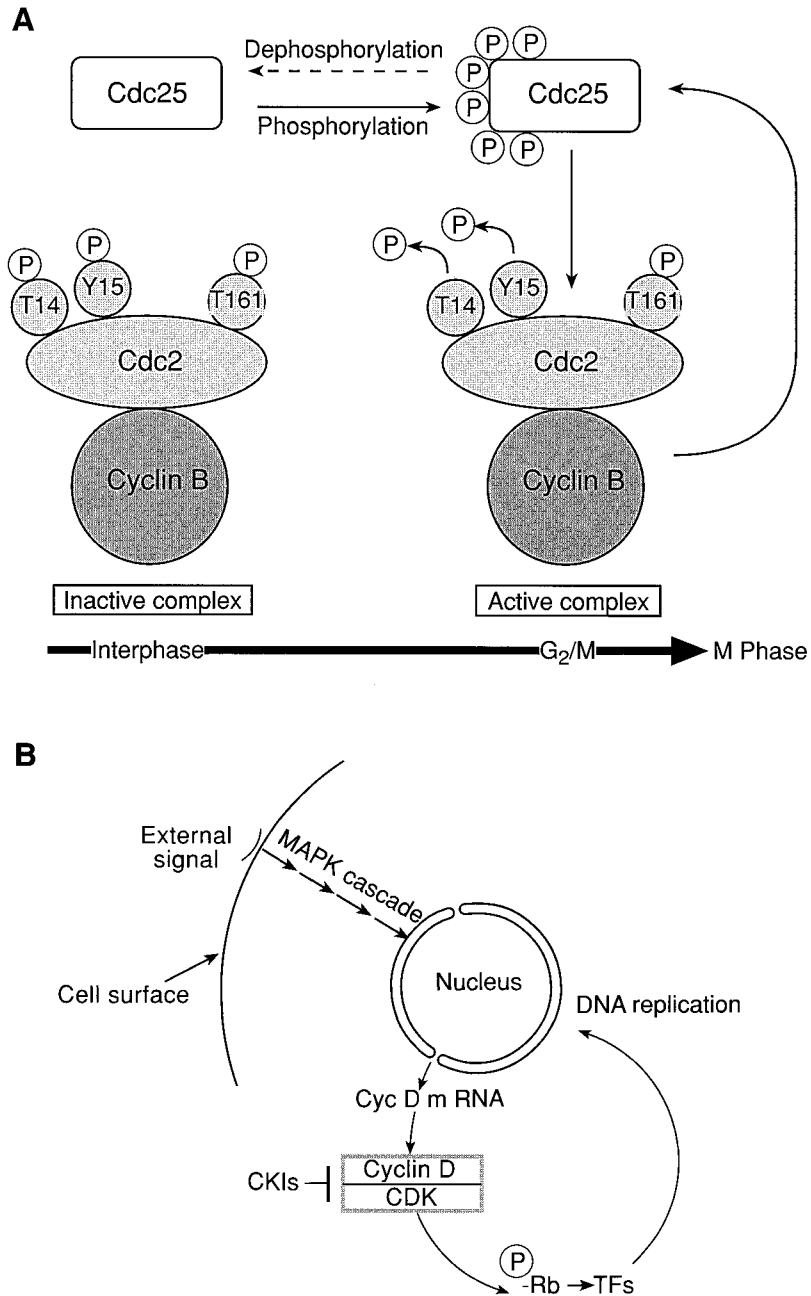


FIGURE 2-28 Two schemes for regulation of the cell cycle. (A) Regulation by phosphorylation and dephosphorylation of conserved amino acid residues in a mitotic cyclin/CDK complex. Cdc25 is a phosphatase which is activated by phosphorylation. In its activated state, it removes the phosphate groups from Tyr-15 and Thr-14, thus activating the Cdc2/Cyclin B complex. Reproduced with permission from Sabelli *et al.* (1998), © Portland Press Ltd. (B) Activation of a cell cycle via the MAPK cascade and its inhibition by inhibitors of the cyclin/CDK complex. A mitogenic signal perceived at the cell surface is transmitted to the nucleus via a MAPK cascade, where it triggers the transcription of *CYCLIN D* RNA. The CyclinD/CDK complex phosphorylates the retinoblastoma protein, which releases the transcription factors (TFs) that, in turn, activate genes for DNA replication. Cyclin kinase inhibitors (CKIs) are proteins that specifically inhibit the kinase activity of cyclin/CDK complexes in G_1 .

4. CYCLINS AND CDKS IN PLANTS

All plants that have been investigated (e.g., carrot, soybean, alfalfa, pea, maize, *Arabidopsis*, *Antirrhinum*, *Sesbania*) have been shown to have cyclins and CDKs. The isolation of cyclins and CDKs often utilizes the technique of functional complementation. This technique has become a very powerful tool to isolate genes

of interest. It requires a suitable vector, usually a haploid yeast, that can be made constitutively defective in a known function and which can then be "rescued" by a protein encoded by a plant cDNA. Insect or human cells have also been used for functional complementation, but the use of diploid organisms is more difficult. We will be referring to this technique at many other places in this book. The technique is outlined in Box 2-3.

BOX 2-3 ISOLATION OF CYCLINS AND CDKS BY FUNCTIONAL COMPLEMENTATION

SACCHAROMYCES CEREVISIAE (BUDDING YEAST) and *Schizosaccharomyces pombe* (fission yeast) are eukaryotic organisms that have many of the genes and proteins and many metabolic pathways and functions that are similar to those in animals and plants. Because these unicellular organisms can be cultured easily under defined conditions and have a short cell cycle, they have proven to be the ideal organism for the study of proteins that regulate cell cycle. More than 100 cell division cycle mutants, most of them described as *cdc* mutants, from yeast are known, and in many cases their wild-type alleles and gene products have been characterized. These mutations are predominantly conditional, i.e., the mutant protein only loses its function under certain conditions, usually at a slightly raised temperature. The cells can therefore be grown at low temperature, but will block in division at higher temperature. The availability of such mutants is of tremendous advantage in isolating cDNAs encoding cell division-specific proteins from other eukaryotes.

Cyclins for the G₁/S transition show much more sequence heterogeneity among species and taxa than mitotic cyclins or CDKs. Thus, routine methods of fishing for them with a cDNA or oligonucleotide probe based on conserved sequences are often unsuccessful. Jim Murray and associates at Cambridge University, Cambridge, UK, used the technique of functional complementation to isolate the D-type cyclins $\delta 1$, $\delta 2$, and $\delta 3$ (henceforth referred to as D1, D2, and D3) from *Arabidopsis* (Sony *et al.* 1995). The technique basically consists of expressing heterologous proteins in a suitable vector that is defective in some particular function. If that function is restored by one of the heterologous proteins, not only is the function of that heterologous protein determined, but the cDNA clone that expressed it can be used to isolate the gene in the original organism.

In budding yeast, three G₁ cyclins are known, whose products accumulate to a threshold, activate the CDC28 kinase, and to transition through the G₁/S checkpoint. Sony *et al.* (1995) inactivated two of these G₁ cyclins and put the third one under a galactose-dependent promoter, such that cells would divide under galactose, but not under glucose. Then used the yeast to express a cDNA library from *Arabidopsis* and selected those clones that allowed the yeast to progress through the S phase and mitosis. These clones represented complementary G₁/S cyclins from *Arabidopsis*. Three cDNA clones encoding, D1, D2, and D3, were obtained and shown to have the greatest sequence similarity to mammalian D-type cyclins.

The technique of functional complementation has also been used to isolate mitotic cyclins and CDKs. Indeed, the first CDK gene isolated in plants was also isolated by functional complementation of a yeast mutant defective in Cdc2 protein (Hirt *et al.* 1991).

4.1. Plant CDKS

CDKs in plants (molecular mass 33–35 kDa) are often given the nonspecific designation *cdc2* because they share as much as 60–65% amino acid sequence identity with *Cdc2* of fission yeast. Plant CDKs show variations in the PSTAIRE and other structural regions, which coupled with their different times of expression indicate that they are as different from one another as the mammalian CDKs (CDK1, CDK2, CDK4, and CDK6). Two types of plant CDKs are recognized. One type carries a conserved 16 amino acid sequence with a-Pro-Ser-Thr-Ala-Ile-Arg-Glu-(PSTAIRE) motif. These CDKs can rescue a fission yeast mutant that is defective in the *Cdc2* protein. The other type has only a partial PSTAIRE motif and its members are not able to rescue a yeast *cdc2* mutant. Both types may be present in the same plant. Thus, snapdragon (*Antirrhinum majus*) has four *cdc2* homologs: *cdc2a*, *b*, *c*, and *d*. While *cdc2a* and *b* carry the PSTAIRE motif, *c* and *d* carry partial motifs. The *Arabidopsis cdc2aAt* carries the PSTAIRE motif and is expressed throughout the cell cycle, whereas *cdc2bAt* carries the PTALRE motif and is expressed preferentially during mitosis. Alfalfa (*Medicago sativa*) has at least six CDKs.

mRNAs of CDKs with the PSTAIRE motif (e.g., *cdc2aAt* in *Arabidopsis*) are present constitutively at low levels in most plant cells that are quiescent, but their levels rise sharply in dividing cells, as in root and shoot meristems, or in pericycle at the time of lateral root initiation. The levels also increase after injury or some environmental or hormonal stimulus. The other type of CDKs have a more patchy occurrence in plants, but their appearance seems to be more closely related to cell division.

Antibodies raised against the PSTAIRE sequence coupled with immunofluorescence have localized some *cdc2* homologues (e.g., *Arabidopsis cdc2aAt*) in association with the preprophase band (PPB) of microtubules in G_2 ; later, during mitosis, the protein disappears, but reappears in association with mitotic spindle and again with anaphase chromosomes. These appearances suggest multiple phosphorylation targets, including cytoskeletal proteins, but proof is lacking.

4.2. Plant Cyclins

cDNAs for more than 60 cyclins have been cloned from a variety of plants, mostly by sequence similarity to known cyclins. A classification and nomenclature system for plant cyclins, based on similarity in primary amino acid sequence and expression patterns to mammalian cyclins, has been proposed and designates them as CycA, CycB, and CycD and their subgroups

as CycA1, CycA2, and so on. A great majority of plant cyclins, more than 50, are mitotic cyclins, which are further divided into CycA and CycB types. Sequence data further subdivide them into five groups: CycA1, A2, A3, and CycB1 and B2. A small number of G_1 cyclins have also been isolated and analyzed. They belong to three subgroups, CycD1, D2, and D3. All three types are known from *Arabidopsis*. A CycD3 cyclin is reported in alfalfa (*Medicago sativa*). Several CycD3 cDNA clones have been isolated from other plants (e.g., snapdragon, tobacco, Jerusalem artichoke, *Chenopodium rubrum*). Cyclins belonging to C, E, or other groups are not yet known from plants.

Cyclins are encoded by small gene families, and several members of the same group (e.g., A or B or D) or subgroup (e.g., A1, A2) may be present in a plant. Individual members seem to be expressed in different tissues/organs and probably in response to different stimuli. D-type cyclins in *Arabidopsis* provide an example. As judged from Northern blots, *CycD3* is highly expressed in roots, is present in lower amounts in flowers, young leaves, and callus tissue, and is absent from stem tissue (Fig. 2-29). *CycD1* produces three transcripts of different sizes. The longest transcript is prevalent in flowers and, to a lesser extent, in roots and callus material, whereas in leaves the intermediate and the shorter transcripts predominate.

As in other eukaryotes, plant cyclins are transcriptionally regulated and expressed in a stage-specific manner only in those cells/tissues that are actually dividing, not those that are capable of division after wounding or some environmental signal. Similar to D-type cyclins in animal systems, *CycD3* mRNA in *Arabidopsis* accumulates rapidly when quiescent cells are presented with growth-promoting substances. Retinoblastoma (Rb)-type proteins are also known in plants and interact with *CycD3* from *Arabidopsis*.

5. CELL CYCLE IN PLANTS HAS SOME UNIQUE FEATURES

Although the mechanisms for G_1/S transition and G_2/M transition are similar in eukaryotes, the triggering events that initiate the entry of cells into cell cycle are unique and specific to different organisms and are governed by their developmental and evolutionary strategies. Quiescent yeast cells are held in a special state of G_1 referred to as G_0 , and specific chemical signals are required to get the resting cells to enter the cell cycle. In yeast, nutrient status and cell size

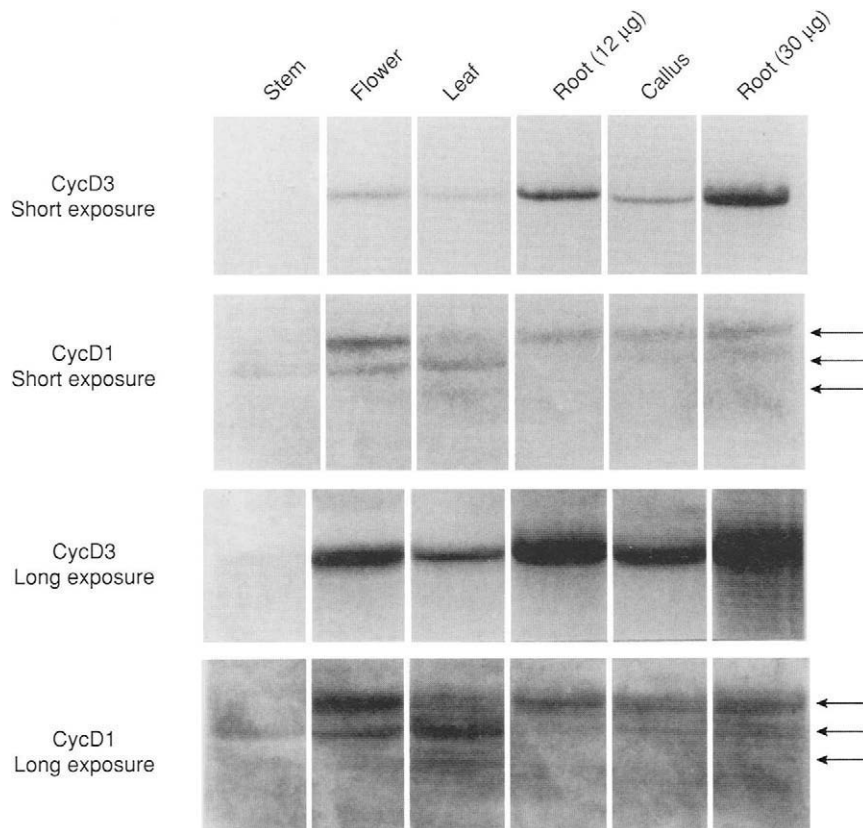


FIGURE 2-29 Expression patterns of *CycD1* and *CycD3* in *Arabidopsis*. Total RNA (30 µg) was loaded in each lane, except lane 4, which received 12 µg. Exposure times were 24 h and 7 days. The longer transcripts in *CycD1* have a larger mass, hence stay closer to the origin than the shorter transcripts. Reproduced with permission from Murray *et al.* (1998), © Portland Press Ltd.

are monitored at a specific time in the G_1 phase, and if they are right, a START signal is given, which initiates the synthesis of G_1 cyclins and the transit of cells to the S phase. When mammalian cells stop dividing, they differentiate and are also held in G_0 . Various internal and external cues, including growth factors, mitogens, antimitogens, and spatial cues, are monitored and integrated at a specific point in the G_1 phase, called the R or restriction point. If the cells go through the restriction point, type D and E cyclins are synthesized and cells go through another round of DNA synthesis and mitosis. Both in yeast and mammalian cells, once the cell cycle is initiated, it goes through the full round to the next G_1 phase (Fig. 2-30A).

In contrast, when plant cells stop dividing, they are held in G_1 or G_2 , even though they may be fully differentiated as a specific cell type (Fig. 2-30B). Thus, the great majority of pericyclic cells in roots are arrested in G_2 . Mesophyll cells in mature leaves of many plants are held in G_1 . In plants, there does not seem to be a comparable state to G_0 , and a precise START or restriction point has not been identified. Also, in plants, the cell cycle,

once initiated, does not need to go through the entire cycle back to G_1 . It can be arrested after S phase in G_2 . For instance, during nodule formation in legumes in response to infection by *Rhizobium* sp., cells of the outer cortex may enter cell cycle and progress from G_1 to G_2 , but then may stop and not progress through the M phase.

These differences in cell cycle between plant cells on the one hand and yeast and mammalian cells on the other may reflect the unique developmental strategies of open growth and open differentiation adopted by plants as a result of their rooted habit (see Chapter 1). It is as if the differentiated but quiescent plant cells are held in a metastable state ready for division should environmental conditions change. In this connection, it is significant that although cyclins are lacking in quiescent cells, mRNAs for CDKs, such as *cdc2* homologues, continue to be present in a constitutive manner in low amounts in most, although not all, plant cells, including nondividing cells. When these cells are induced to divide by wounding, or environmental or hormonal stimulus, the levels of their mRNAs increase sharply, as do the levels of their cognate cyclins.

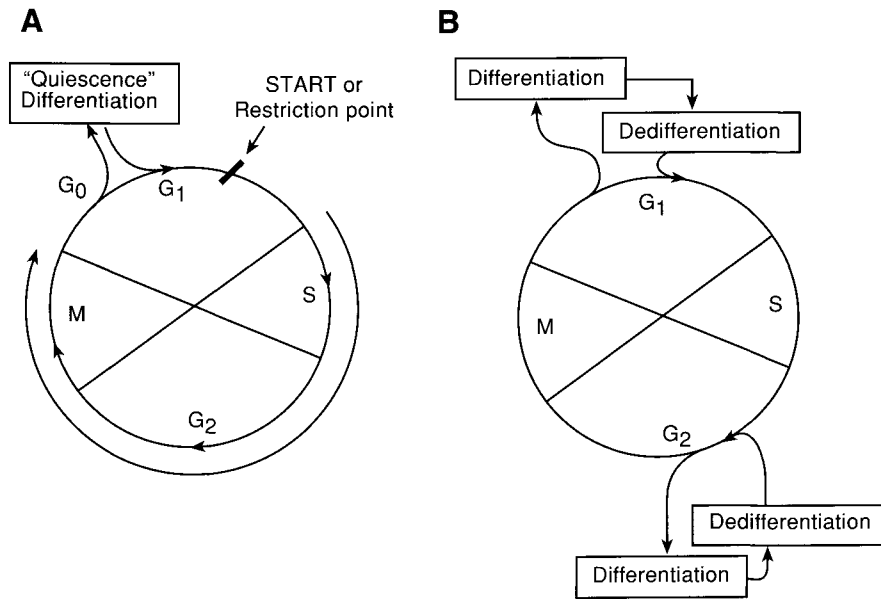


FIGURE 2-30 Cell cycle in yeast and mammals (A) and in plants (B). In yeast and mammalian cells (A), the G₁/S transition is regulated by the checkpoint called start or restriction point, respectively. In plants (B), no such specific point has been discovered to date. Once past the start or restriction point, yeast and mammalian cells usually go through the entire round of cell cycle. In contrast, plant cells may be held in either G₁ or G₂. Moreover, they may enter the differentiation or dedifferentiation pathway from either G₁ or G₂. Modified from Ferreira *et al.* (1994) with kind permission from Kluwer.

In summary, many cyclins and CDKs have been isolated from plants and their expression patterns described, but their specific roles during cell cycle are still far from clear. Cyclins in general are better characterized, but it is not known whether different CDKs are required for the G₁/S or G₂/M transition or which cyclins associate with which CDKs. Also unknown are the natural substrates of CDKs. Biochemical purification of cyclin-CDK complexes from actively dividing tissues and the subsequent determination of their subunit composition, as also the cellular localization patterns of different cyclins and CDKs, would be of help. Environmental factors, such as light, wounding, stress situations, and treatments with plant hormones, are known to trigger cell divisions in plants. That subject is covered in Chapter 15. Homologues of CKI and Rb-type proteins have been identified in plants and shown to interact with plant D-type cyclins.

6. PLANES OF CELL DIVISION

Planes of cell divisions are occasionally random, but very often they are highly oriented (Figs. 2-31A and 2-31B). In the rib meristem below the shoot apex and in the intercalary meristem at the base of grass leaves, they occur at right angles to the long axis of the organ,

resulting in longitudinal files of cells; in tunica layers in the shoot apex, they are anticlinal, except where leaf primordia are initiated, when they are periclinal. Fusiform initials in cambium are a model *par excellence* of oriented divisions. In conifers, they may be up to 10 mm long (e.g., redwood, *Sequoia sempervirens*) and about 10 × 40 μm in cross-sectional diameter, yet these cells divide tangentially (periclinally) along their entire length to give rise to a xylem or a phloem derivative. Mitosis occurs in the middle of the cell, and the new cell plate, initially only a few micrometers in length, grows longitudinally up and down to complete the cytokinesis (Figs. 2-31C–E). Periodically, the fusiform initials also divide anticlinally to give rise to two daughter initials.

Cell divisions may also be symmetric or asymmetric. In symmetric divisions, the parent cell is partitioned into two nearly equal halves. Most cell divisions are symmetric divisions (Fig. 2-32). In several cases, however, cell division is asymmetric and results in the production of two daughter cells that are vastly different in size as well as cytoplasmic complement. During the formation of stomata in grass leaves, an epidermal cell divides to produce a small guard cell mother cell (GCMC) and a larger cell, called the "pavement" cell (Fig. 2-32). The GCMC divides longitudinally and the products differentiate as two guard cells.

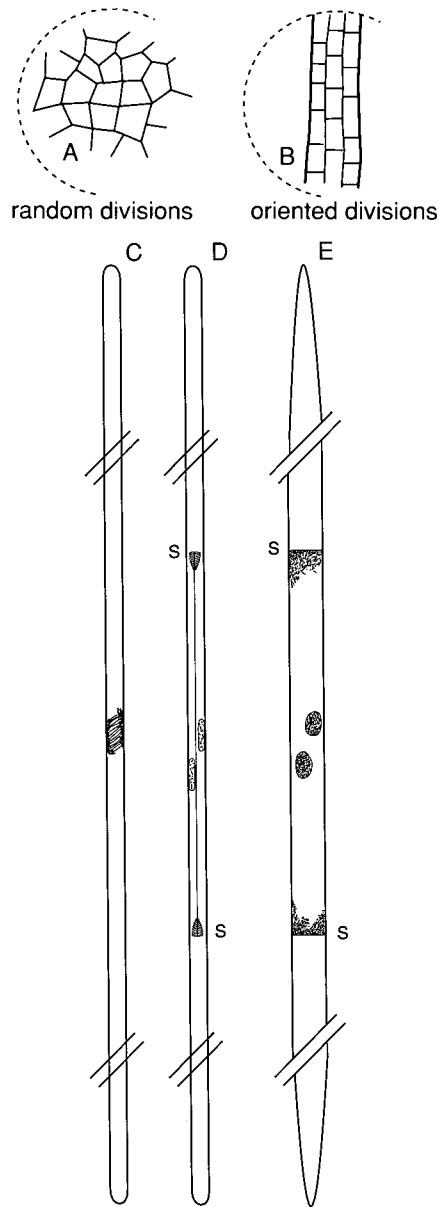


FIGURE 2-31 Diagrammatic representations of planes of cell divisions. (A) Random planes of divisions. (B) Oriented transverse divisions that give rise to longitudinal files of cells. (C–E) Periclinal (tangential) division in a fusiform initial in the cambium of a conifer. Mitosis occurs in the middle of the cell (C). The new cell plate is initiated there and then grows longitudinally (D) until it reaches the two ends of the cell. C and D are views of the initial in a radial longitudinal section, and E is the same as D, but seen in a tangential longitudinal section. C–E from Bailey (1954).

The neighboring pavement cells, however, undergo additional asymmetric divisions, one each, to produce a small lens-shaped subsidiary cell next to each guard cell. There are many other examples of asymmetric divisions; they include formation of root hair in grass roots, formation of companion cells from sieve tube

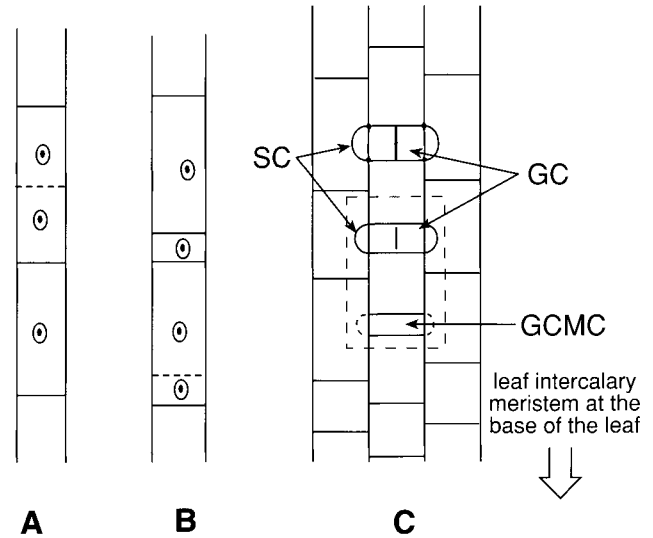


FIGURE 2-32 (A) Symmetric division. (B and C) Asymmetric divisions in formation of the stomatal complex in grass leaves. A small cell [guard cell mother cell (GCMC)] is cut off and divides longitudinally to give rise to two guard cells (GC). The neighboring pavement cells also divide asymmetrically to produce subsidiary cells (SC). The area marked in C by a dashed rectangle is referred to in Fig. 2-35.

mother cells in phloem tissue of angiosperms, and formation of generative cells in angiosperm pollen.

Before we consider how planes of cell divisions are regulated, we must understand how cytokinesis occurs in plants.

7. CYTOKINESIS IN PLANTS

While mitosis occurs in plants in basically the same manner as in animals, cytokinesis follows an entirely different course. In animal cells, cytokinesis starts at the plasmalemma as an acto-myosin-based constriction, which proceeds inward bisecting the cell. In plants, the cell plate starts in the center of the cell and grows outward until it meets the neighboring walls. In late anaphase/early telophase, small Golgi vesicles begin to move toward and aggregate at the cell equator, the interface between the two sets of mitotic spindles. Small tubular outgrowths from the margins of these vesicles fuse, forming a tubulo-vesicular network (Fig. 2-33). With continued traffic and fusion of vesicles, this network begins to flatten and extend centrifugally as the nascent cell plate. This centrifugal movement is aided by the shortening of the microtubules (MTs) in the phragmoplast accompanied by their lateral extension.

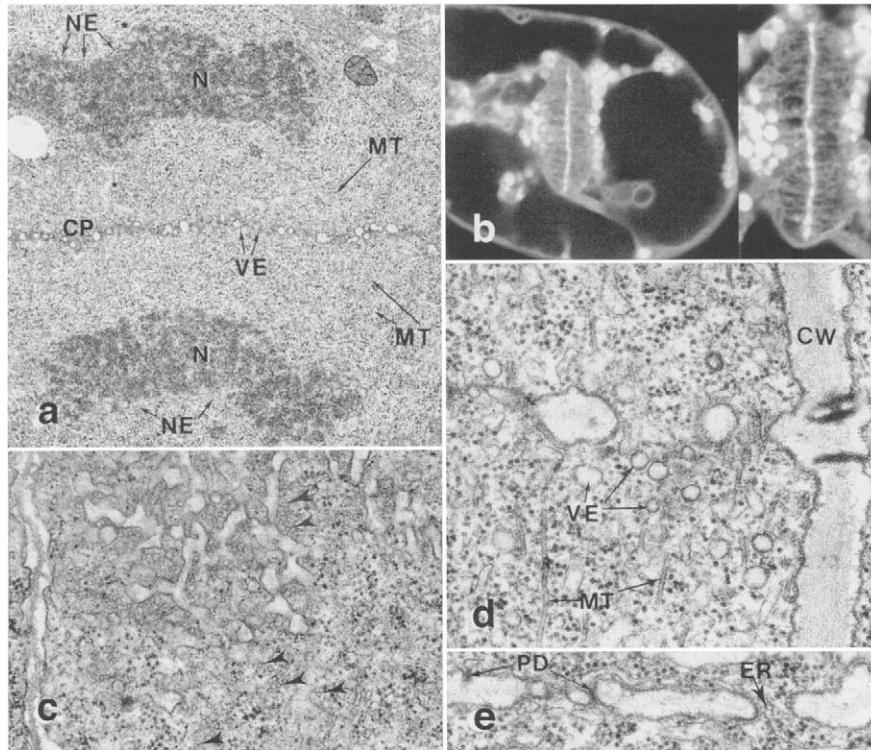


FIGURE 2-33 Light (b) and transmission electron (a,c,d,e) micrographs showing cytokinesis in plant cells. (a) Two daughter cells with telophase nuclei and the developing cell plate (CP). Golgi vesicles (VE) are discrete, but some are beginning to coalesce to form larger units. *Beta vulgaris* root tip. $\times 7,800$. (b) Light micrographs of a tobacco cell in culture (line Bright Yellow-2 or BY-2) showing formation of the cell plate. The living cell was stained with rhodamine-123, a vital stain. The cell plate, membranes, and some organelles stain brightly; the telophase nuclei are out of the plane of focus. $\times 1100$; inset $\times 1800$. (c) The cell plate in face view, at right angles to the plane in (a). The plate has an undulating contour, hence passes into and out of the plane of the section. Phragmoplast microtubules are seen in cross-sectional view (arrowheads). *Avena sativa* anther. $\times 27,000$ (d and e) Small segments of cell plates showing the presence of ER through a gap between fusing vesicles, which will eventually form a plasmodesma with a desmotubule, and point of contact of the extending cell plate and the side wall of the dividing parental cell. Both from *Vicia faba* root tip; $\times 31,000$. From Gunning and Steer (1996).

The nascent plate continues to grow centrifugally until it reaches the cell margin; once there, many finger-like processes project out and fuse with the plasmalemma of the parent cell, thus creating two daughter cells. The fused vesicle membranes become the new plasmalemmae, and the fibrillar material inside becomes the new intercellular matrix. Gaps between fusing Golgi vesicles become future plasmodesmata. They are lined with plasmalemma and usually harbor an ER tubule, known as desmotubule. The plasmodesmata provide for intercellular communication and transport of materials.

Golgi vesicles carry wall materials typical of matrix polysaccharides, pectins and hemicelluloses, and the new cell plate is the middle lamella. Cellulose synthesis follows closely and is accompanied by elaboration of the primary wall. Chemicals that inhibit cellulose or wall synthesis (e.g., herbicide 2,6-dichlorobenzonitrile,

which blocks cellulose synthesis reversibly) disrupt cytokinesis without disrupting mitosis and lead to bi- or multinucleate cells.

7.1. Control of the Plane of Cell Division

The cellular agencies that control the plane of cell division are unknown, but plasmalemma and certain elements of the cytoskeleton, specifically microtubules, are involved directly or indirectly in specifying the location of the future cell plate. Earlier studies utilized transmission electron microscopy of chemically fixed and thin-sectioned plant materials. In more recent years, these studies have been supplemented with immunofluorescence studies, utilizing antibodies against tubulin or actin linked to fluorescent dyes to localize MTs or actin filaments, respectively, in living

or chemically fixed cells (see Box 2-4). Also, drugs such as colchicine and cytochalasin B, which bind to depolymerized monomers tubulin and G-actin and prevent their polymerization into MTs or fibrillar actin, respec-

tively, and drugs such as taxol, which stabilize the polymerized MTs and prevent their disaggregation, have been used to study the interaction of MTs and/or actin filaments with cell division.

BOX 2-4 VISUALIZATION OF THE CYTOSKELETON: IMMUNOFLOUORESCENCE OF MICROTUBULES AND MICROFIBRILS

MUCH OF THE WORK in the 1960s and 1970s on the structure of cell walls and MTs was based on careful observations on chemically fixed and thin-sectioned materials under the transmission electron microscope. These studies provided a wealth of information on the orientation of cellulose fibrils and cortical microtubules, which up to that time was impossible to achieve by any other means, but of necessity they looked only at small segments of the material. The availability of antitubulin antibodies and their visualization by fluorescein-labeled anti-antibodies in early 1980s opened a way for studying microtubular distribution and orientations in whole cells under the light microscope. Subsequent improvements in these techniques, as well as the advent of confocal laser-scanning microscopy, coupled with computer graphics, now allow visualization of microtubules in living cells. In the following, these immunofluorescent techniques are described.

In earlier studies, strips or sections of fresh tissues were chemically fixed with an aldehyde (usually paraformaldehyde) in buffer, washed, and treated with wall-digesting enzymes (e.g., Onozuka cellulase / macerozyme mixture) for a short time. They were washed, air dried, and stained with an antibody against α - or $\alpha\beta$ -tubulin, then washed again, and counterstained with a fluorescein-conjugated anti-antibody. If the material needed to be examined in a particular plane of section, fixed material could be frozen, 60- to 80- μ m sections obtained on a freezing microtome, then thawed, and used as just described. Cell walls could be hydrolyzed, depending on need. Examination was under an ordinary light microscope or an epifluorescence microscope. While providing very graphic information on the arrangement of MTs in whole cells, these studies nonetheless suffered from poor imaging and resolution because of the backscatter of fluorescence. The invention of the confocal laser scanning microscope (CLSM) was a signal achievement. In a CLSM, a laser beam serves as the light source. The laser light is focused to a point and is used to scan the specimen in a raster-like pattern. The elegance of a confocal microscope is that it can be used to optically section the specimen in as little as 1- μ m steps by changing the focal length of the beam. In one stroke it eliminates background scatter and, at the same time, provides a three-dimensional representation of the object by pooling information from different focal planes. Linking of a CLSM to a graphics computer enables not only storage of images, but their integration and display.

With improvements in imaging provided by CLSM, preparation of the material has been simplified. A major help in this area was provided by the discovery that heterologous tubulin from pig or cow brain injected into plant cells behaves as if it were of plant origin—it becomes incorporated into the endogenous pool. A stain, rhodamine, which is fluorescent when excited with a laser beam, can be conjugated to such tubulin and acts as a reporter. Thus, in a one-step process, plant cells can be injected with a mixture of heterologous tubulin and rhodamine and prepared for observation under the CLSM.

Other modifications, such as fluorescence redistribution after photobleaching, allow study of the turnover of tubulin monomers and MT assembly.

Similar studies, such as those on microtubules, can be conducted on actin fibrils or microfibrils. In this case, antiactin antibodies and specific stains that bind to actin, such as phalloidin, are used.

Fluorescence microscopy using CLSM has also proven very useful for visualizing the distribution of calcium (Ca^{2+}) in the cytosol and other intracellular compartments. To visualize calcium, calcium-binding dyes, which fluoresce in visible light on excitation by UV (340 or 380 nm), are microinjected into the cell, or introduced in some other manner, and provide information on the distribution of Ca^{2+} within the cell.

Despite its manifest advantages, CLSM does have a few drawbacks. The choice of specific fluorescent dyes is limited to those that can be excited by the available lasers. Photobleaching of dyes and cell damage by laser are other drawbacks.

7.1.1. The Plane of Cell division Is Marked by PPB of Microtubules

A typical plant cell in interphase usually shows an array of MTs in the cortical cytoplasm. Prior to prophase (late in S phase or early in G₂), this cortical array begins to disappear from most of the cell and, instead, becomes concentrated as a distinct band in the form of a ring or girdle. This band, called the preprophase band (PPB) of MTs, appears well before the beginning of mitosis and marks the location where the future cell plate is going to be formed. The PPB band was first discovered in connection with asymmetric cell divisions in wheat leaf cells in the late 1960s (Pickett-Heaps and Northcote, 1966). Since then, the PPB has been shown to be a regular feature in almost all plant cell divisions, asymmetric or symmetric, in intact tissues, and even in single cells in culture.

The PPB is usually well formed during G₂. With the breakdown of the nuclear envelope and condensation of chromosomes at the beginning of prophase, the PPB begins to disappear and instead MTs reappear as mitotic spindle fibres. As karyokinesis is completed, during telophase, new arrays of MTs appear at the phragmoplast (a complex structure composed of ER, fusing Golgi vesicles, and phragmoplast MTs) and guide the new cell plate toward the cell periphery. These various incarnations of MTs in the cell are shown graphically in Fig. 2-34, which shows wheat root tip cells in the process of cell division. After the cell division is complete and the spindle and phragmoplast MTs disappear, new arrays of MTs appear again in the cortical cytoplasm.

What is remarkable is that the new cell plate is oriented in the plane previously marked by the PPB. This predetermination of the location of the new cell plate by the PPB is even more graphically illustrated in those cells where unequal or asymmetric divisions occur. For example, in the formation of subsidiary cells in grass leaves, the new cell wall is curved (Fig. 2-35). The PPB, likewise, occur in a curve, like an ellipse around the mother cell.

The close parallelism between the site of PPB assembly and the future cell plate suggests that plant cells establish their division plane before entering mitosis. The internal and external factors involved in PPB assembly and site marking are unknown, but there is evidence for phosphorylation and/or dephosphorylation of some proteins in that milieu. Indirect immunofluorescence using primary antibodies raised against a conserved sequence in a cdc2 kinase of maize (a homologue of p34cdc2) shows colocalization of the CDK with PPB in root tips and stomatal complex cells in grasses (Fig. 2-36). In more recent years, a mitotic

cyclin, CycB1, in maize has also been colocalized at the PPB, but a functional complexing of the two proteins, i.e., CycB1 and CDK, is not predicted.

Evolutionarily, the PPB are associated with land plants, i.e., vascular plants and bryophytes; they are absent in algae and fungi. Remarkably, they are also absent in higher plants in cells that divide meiotically or are involved in sexual reproduction. Thus, they are absent in the formation of the embryo sac, pollens, and the male gametes, but they reappear with the first division of the zygote.

SECTION III. CELL GROWTH IN PLANTS

Plant cells show two basic types of growth: tip growth and overall growth or diffuse growth (Fig. 2-37). The mechanisms and the requirements for the two types of growth are vastly different.

1. TIP GROWTH

Tip growth is seen in several types of cells, including root hairs, pollen tubes, intrusive growth of differentiating fibers and sclereids, and of fusiform initials in nonstoried cambia and in germinated fern spores (fern protonemata). Tip growth permits tortuous growth or changes in the directionality of growth, e.g., when root hairs encounter soil particles or when pollen tubes traverse the female tract in their passage to the embryo sac.

Pollen tubes have become a model system to study tip growth because pollen grains are available in large quantities, they can be germinated in a drop of nutrient solution and growth of the tubes can be monitored *in vitro*. This section describes tip growth in pollen tubes. The tip of the pollen tube shows a clear zone, which is rich in vesicles and some membranes, but relatively free of other organelles. The vesicles fuse with the plasma-lemma at the tip and provide membrane and wall materials for elongation (Fig. 2-38). In the more distal parts, away from the tip, pollen tubes show rapid cytoplasmic streaming, especially in the central, more fluid, part of the tube and, associated with it, numerous bundles of microfibrils (F-actin). Microtubules, rough ER, dictyosomes, and other organelles, including the vegetative nucleus and the two male gametes, occur in the distal nongrowing regions of the tube (Fig. 2-38) and do not seem to be involved directly in tip growth. These cytoplasmic contents keep moving with the growing tip, as the older, distal parts of the tube get cut off by periodic deposits of callose, a β -1, 3-glucan.

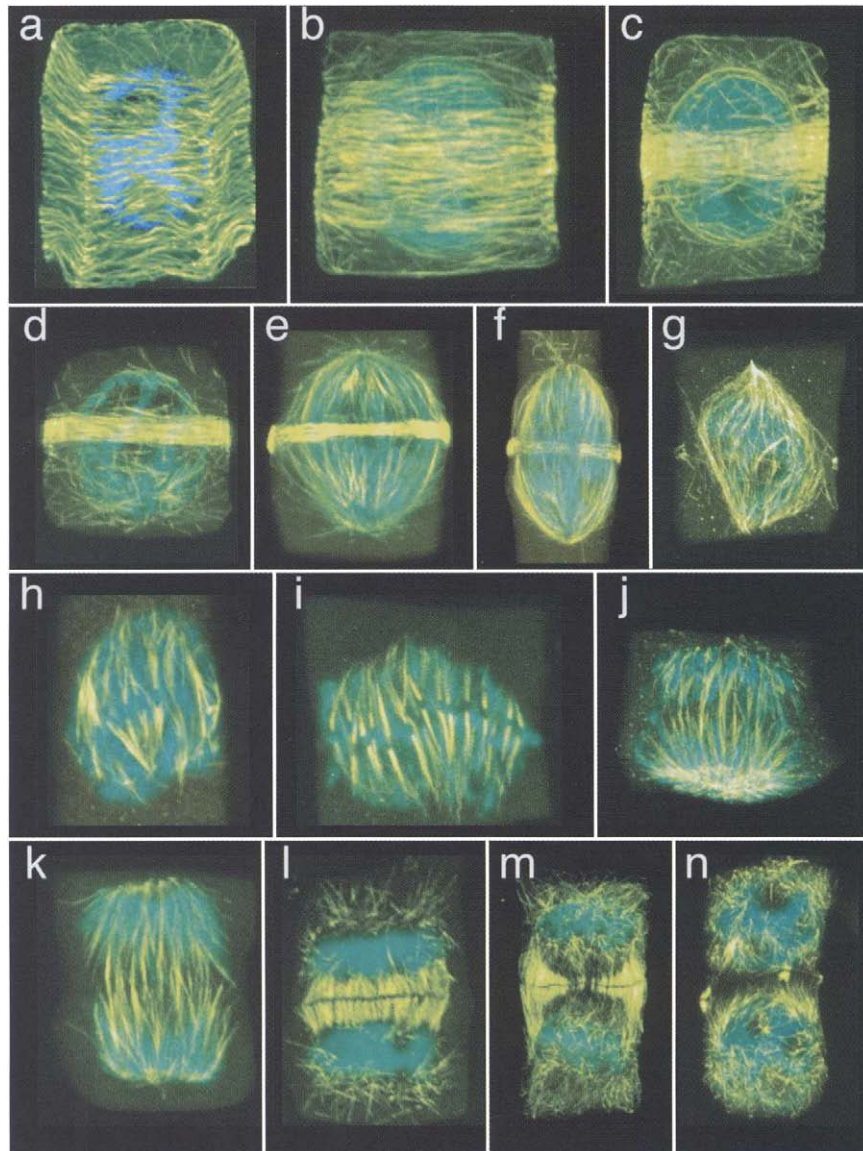


FIGURE 2-34 Microtubules during interphase and the cell division cycle. Cells from wheat root tips were stained using antitubulin antibody. MTs appear yellow, whereas chromatin or nuclei appear blue. Photographs were taken using a confocal scanning microscope. a, shows an interphase cell, the cortical MTs occur mostly along the longitudinal walls and show a transverse orientation; b and c, preprophase—stages in the aggregation of PPB, starting after the end of S phase and culminating as the PPB band; d, g, and h, prophase—the mitotic spindle starts to form, the chromosomes continue their condensation, the PPB gradually erodes away, and the nuclear envelope breaks down; i and j, prometaphase-anaphase—bundles of MTs attach to the kinetochore regions of the chromosomes, separation of chromatids occurs and one set is moved to each pole; k and l, telophase and phragmoplast development—cell plate begins to show up as a narrow nonfluorescent zone at the midline of the phragmoplast. Note the alignment of the new cell plate with the earlier location of the PPB; m and n, MTs disappear from the phragmoplast as the cytokinesis is completed, and gradually reappear in the cortical cytoplasm. Magnifications of photographs range from $\times \sim 780$ to 1250. From Gunning and Steer (1996).

The composition of the pollen tube wall is still not fully understood. The constituents at the tip include hemicellulosic polymers, such as arabinans, but not cellulose. The distal, nongrowing parts show cellulose

and also appreciable amounts of pectins, hemicelluloses, especially arabinans, and callose, which may be present as a separate inner layer in the wall. Glycoproteins are also present.

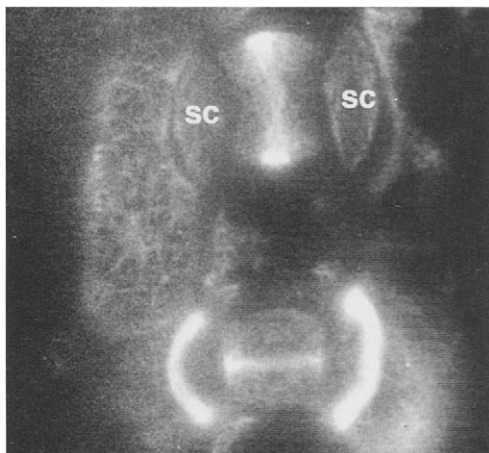


FIGURE 2-35 Segment of rye leaf epidermis containing developing stomatal complexes. A lens-shaped subsidiary cell (sc) appears on either side of the guard cell mother cell at the top, which has a longitudinal PPB. In the developmentally younger cells below these, PPBs mark the forthcoming division of each of the subsidiary mother cells. Note that the curved PPBs accurately predict the placement of the wall that outlines the subsidiary cells. The lower guard cell mother cell is still in interphase and shows a characteristic transverse band of MTs. The photograph corresponds approximately to the area marked by a dashed rectangle in Fig. 2-32 ($\times 1200$). Courtesy of Soon-Ok Eun and Susan Wick; original photograph from Wick *et al.* (1989).

The motive force for tip growth is not clear. Some authors believe that tip growth requires turgor pressure, whereas others state that it can occur in the absence of osmotic potential and does not require water uptake. The motive force for growth could be provided by the bundles of actin fibrils, or microfilaments, which are axially aligned, but they usually do not extend into the clear zone. They do provide the motive force for cytoplasmic streaming, however. Cytochalasin B, which depolymerizes actin filaments, stops cytoplasmic streaming and also tip growth.

Calcium ions play an important role in pollen tube growth. Not only are they essential for growth to occur, they also are involved in modulating the directionality of growth. In addition, they are also reported to direct the flow of Golgi vesicles carrying wall polysaccharides and membrane material to the growing tip. Levels of intracellular calcium can be measured using fluorescent dyes that bind to calcium, and measuring fluorescence by suitable microscopic techniques (e.g., epifluorescence or confocal laser scanning microscopy; see also calcium signaling, in Chapter 25). Using these techniques, it has been shown that the concentration of free calcium in the cytoplasm, $[Ca^{2+}]_c$, not the calcium that may occur in vacuoles or cell wall, is very high at the tip. It can be as high as $2\text{--}3\text{ }\mu\text{M}$ at the apical dome and falls quickly to basal levels of about $0.2\text{ }\mu\text{M}$ (about 200 nM) within $20\text{--}30\text{ }\mu\text{m}$ of the tip apex. This is roughly the clear

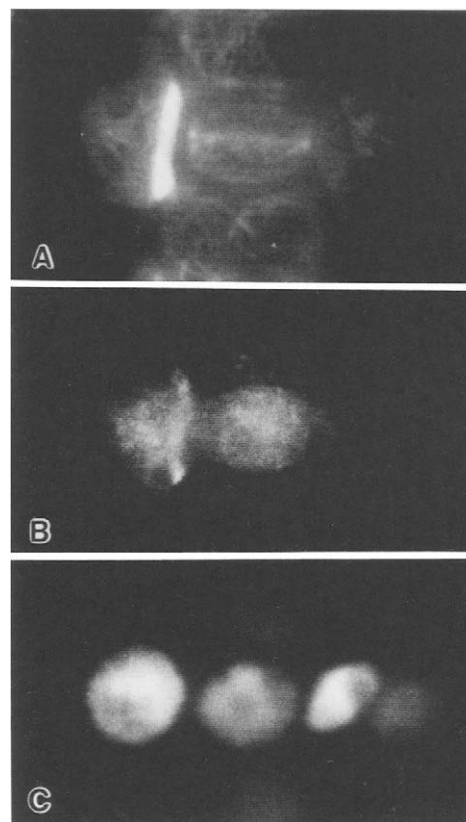


FIGURE 2-36 Immunofluorescence staining of maize leaf epidermis showing a developing stomatal complex. The same stomatal complex cells are shown in A–C. (A) Cells stained with tubulin antibody, which reveals the transverse interphase band of MTs in the guard cell mother cell (GCMC) and the more brightly stained PPB in the subsidiary mother cell (SMC) on the left. (B) The same complex stained with maize p34^{cdc2}Zm antibody. Note that the CDK colocalizes with PPB in the SMC, but not with the interphase MTs in GCMC. (C) Cells stained with DAPI, a specific stain for DNA. Magnification $\times 1600$. From Colasanti *et al.* (1993).

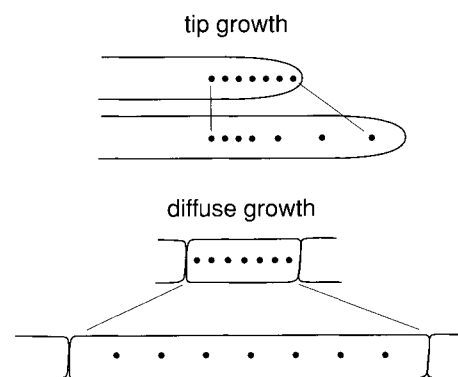


FIGURE 2-37 Tip growth and overall growth as shown by markings with India ink. In tip growth, growth occurs only at the tip of the cell; the rest of the cell does not grow. In surface growth, growth occurs over the entire organ; all its constituent cells grow in concert.

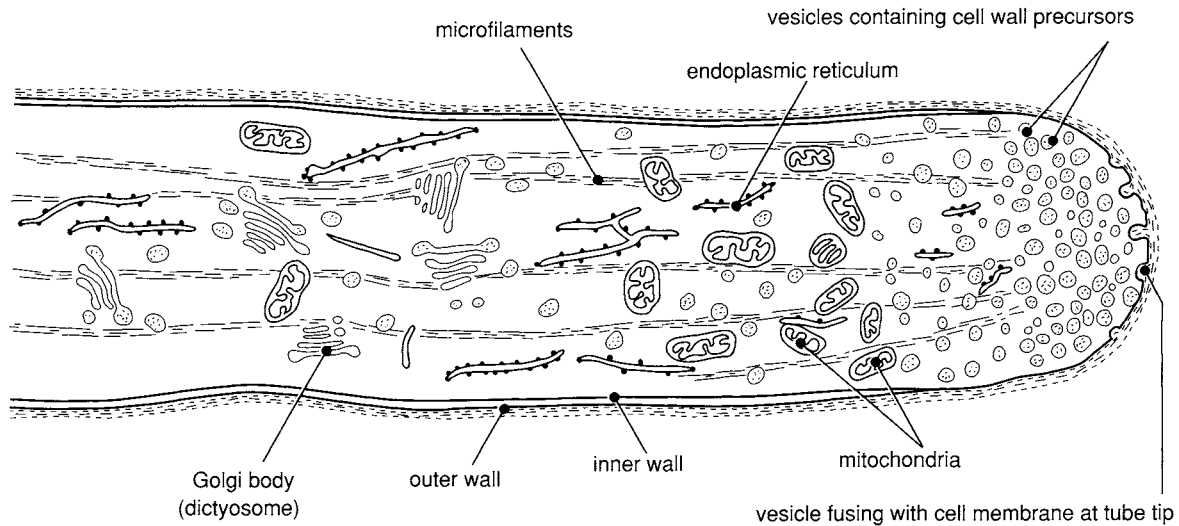


FIGURE 2-38 A diagrammatic representation of a growing pollen tube. In the apical tip part of the pollen tube, vesicles deliver membrane and wall materials to the growing tip by exocytosis. The distal part of the pollen tube shows ER, other organelles, and bundles of microfibrils. From Mascarenhas (1993).

zone seen at the apex. The high concentration at the tip is maintained by a constant flow of Ca^{2+} from the external medium into the tip via specific calcium channels; dissipation at the distal end is accomplished by pumping out of calcium by Ca^{2+} -specific ATPases (see Box 13-1, Chapter 13). The intracellular tip-focused calcium gradient, which dissipates quickly in the absence of calcium influx from outside, is necessary for pollen tube growth. Buffers that dissipate the gradient, microinjection of chelators that bind cytosolic calcium, or drugs, such as caffeine, that disrupt the tip-focused gradient stop pollen tube growth, and growth is not resumed until after the gradient is reestablished. It is noteworthy that caffeine, while stopping tip growth, does not disrupt microfibrils and cytoplasmic streaming.

Calcium also seems to be involved in changing the orientation of growth. Calcium ions can be caged in molecular baskets (ionophores), which can then be injected into the cell. Inside the cell, caged calcium can be released by photolysis of the basket; thus, raising the level of calcium in a localized area of the cytosol. Using this technique, it has been shown that pollen tubes bend, or a new growth center is initiated, toward the side where the cytosolic calcium level has been raised (Fig. 2-39). Calcium is a divalent cation, and its effects can be mimicked by an electric field. Such fields disrupt the orientation of tube growth, and the tube grows toward the cathode. Although these *in vitro* experiments provide clear evidence for a role of cytosolic calcium in pollen tube growth and control of its directionality, it is not yet clear whether the same phenomenon pertains to pollen tube growth *in vivo*.

Root hair growth has not been studied to the same extent, but here also a tip-focused calcium gradient has been reported.

2. DIFFUSE OR OVERALL GROWTH

All plant organs that show elongation growth, e.g., subapical parts of stems and roots, grass coleoptile, intercalary region at the base of grass leaves, petioles, and inflorescence axes, show a diffuse or overall growth, which involves not a single cell, but hundreds and thousands of cells, some of different wall characteristics, which all grow in concert, cemented as they are by the intercellular matrix (see Fig. 2-37). Diffuse growth is also seen in internodal cells of some green algae belonging to Characeae (e.g., *Nitella* and *Chara*). These cells are not constrained by neighboring cells; hence, they have provided a unique material for the study of diffuse growth. The diffuse growth of stems and coleoptiles is affected by hormones and, hence, is covered in Chapter 15 after we have dealt with the structure and metabolism of hormones and their uptake and transport in the plant body. However, it is important to emphasize here that the mechanical requirements for diffuse growth are very different than those for tip growth. First, this type of growth involves wall loosening in all the constituent cells, and the walls must be loosened in their entire thickness. Second, there must be a motive force for cell expansion; this motive

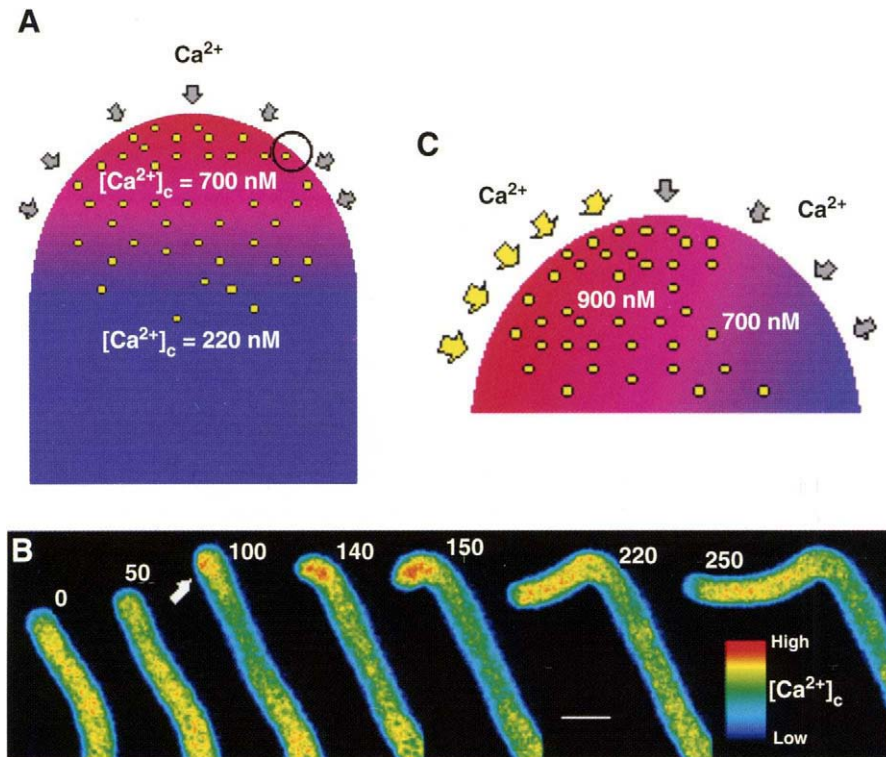


FIGURE 2-39 Calcium gradient at the tip of a pollen tube and change in orientation of growth in response to an asymmetric increase in cytosolic calcium. (A) The gradient is maintained by the entry of calcium ions at the tip of the pollen tube and dissipation at sites away from the tip. The gradient is responsible for the fusion of Golgi vesicles (small spherical bodies) at the tip and, hence, growth of the pollen tube. (B) Calcium movement during pollen tube reorientation. A time course series of confocal images indicating Ca^{2+} movement after the release of caged Ca^{2+} in the left hemisphere (see C) of the apical zone at ~ 95 s is shown. The times (in seconds) at which images were taken are shown adjacent to the growing tube. Color images are coded according to the scales shown. Bar: $10\mu\text{m}$ (C) Detail of the pollen tube tip after the release of caged calcium in the left hemisphere. From Trewavas and Malhó (1997).

force is provided by the uptake of water. Third, it has often been observed that as the cells grow, their wall thickness is maintained, which means that as walls are loosened and the cells grow, new cell wall material is deposited in the walls. Fourth, there must be a mechanism to control the directionality of growth, i.e., whether the cells grow longitudinally, transversely, or radially.

SECTION IV. CYTOSKELETON PLAYS IMPORTANT ROLES IN CELL GROWTH, CELL SHAPE, AND CELL DIFFERENTIATION

1. DIRECTIONALITY OF OVERALL CELL GROWTH

Whereas microtubules are not directly implicated in tip growth, they play an important role in overall cell

or diffuse growth. As mentioned earlier, they seem to regulate the orientation of newly deposited cellulose fibrils. In all cases studied, there is a strong correlation between the orientation of MTs and new fibrils on the one hand and the directionality of growth on the other. In longitudinally elongating cells, the orientation of MTs and the newly deposited fibrils is transverse to the long axis; in cells that are expanding transversely, it is oblique to longitudinal to the long axis. In cells that expand radially in all directions, the orientation is random. The directionality of growth of constituent cells determines the direction of organ growth.

These correlations are further supported by treatments with chemicals that result in the depolymerization of MTs. Colchicine binds to tubulin monomers and prevents their aggregation to form MTs. Thus after colchicine treatment, most of the tubulin occurs in the monomer pool and no organized MTs are left in the cell. If colchicine is given to cells that are not enlarging, MTs dissolve, but there is no *detectable* effect

on the orientation of cellulose fibrils in the wall. However, if colchicine is given while cells are boosted to grow by hormonal treatment, MTs are dissolved, the new wall fibrils show a random arrangement, and the cells show an enormous isodiametric expansion.

Whether the orientation of innermost wall fibrils and cortical MTs is the underlying factor responsible for the direction of cell expansion or is a consequence thereof is not known.

2. DETERMINATION OF CELL SHAPE

Plants show some unusual structures, such as glandular trichomes on stems and leaves (e.g., leaf of maple, *Acer* sp.), the pitcher that develops from the leaves of many species of pitcher plants and serves as the trap for insects, and the flower of an orchid (Fig. 2-40). These structures acquire their specific form and shape because the constituent cells divide in specific planes and show differential growth, as well as differential wall deposition.

In many cases, individual cells also acquire specific shapes, e.g., palisade mesophyll in leaves, guard cells in stomata, idioblasts, and sclereids. They acquire different shapes by a combination of growth coupled with selective restraint at places, which subsequently is reinforced by the deposition of wall materials. For example, in wheat leaves, mesophyll cells at maturity resemble tube-shaped balloons with almost regularly spaced constrictions. The constrictions arise by the

selective deposition of wall material at “hoop” sites where MTs provide the scaffolding. This initial patterning by MTs is important. After wall deposition at constriction sites has occurred, the MTs may be more evenly distributed and the relatively thin walls of the bulges are uniformly reinforced (Fig. 2-41). MTs may also disappear after cell shaping is complete.

3. DIFFERENTIATION OF TRACHEARY CELLS

Tracheary cells, i.e., tracheids and vessel elements in xylem, are a model system for cell differentiation. During primary growth in stems and roots of angiosperms, the putative vessel elements undergo precise changes in four continuous but well-defined stages: (i) the cells expand in diameter and elongate severalfold over their precursor procambial cells; (ii) the cells deposit a secondary wall in precise patterns over parts of the primary wall, leaving other parts uncovered; (iii) lignin is deposited in the walls, except where the primary walls are left exposed; and (iv) the cells commit “suicide” (undergo apoptosis)—the entire cytoplasm, the nucleus, and parts of the primary wall left exposed and unlignified are hydrolyzed. There is a minor but evolutionarily significant variation in tracheids, the other type of tracheary cells, which are the predominant type present in gymnosperms and ferns. In tracheids, in the final stage of hydrolysis, the primary wall that is left uncovered by the secondary wall is not dissolved but is left intact.



FIGURE 2-40 Some elegant forms in plants. (A) Orchid Flowers. (B) The Cobra plants (*Darlingtonia californica*), one of a group of insectivorous plants from different families that form pitcher-like structures at the distal ends of their leaves.

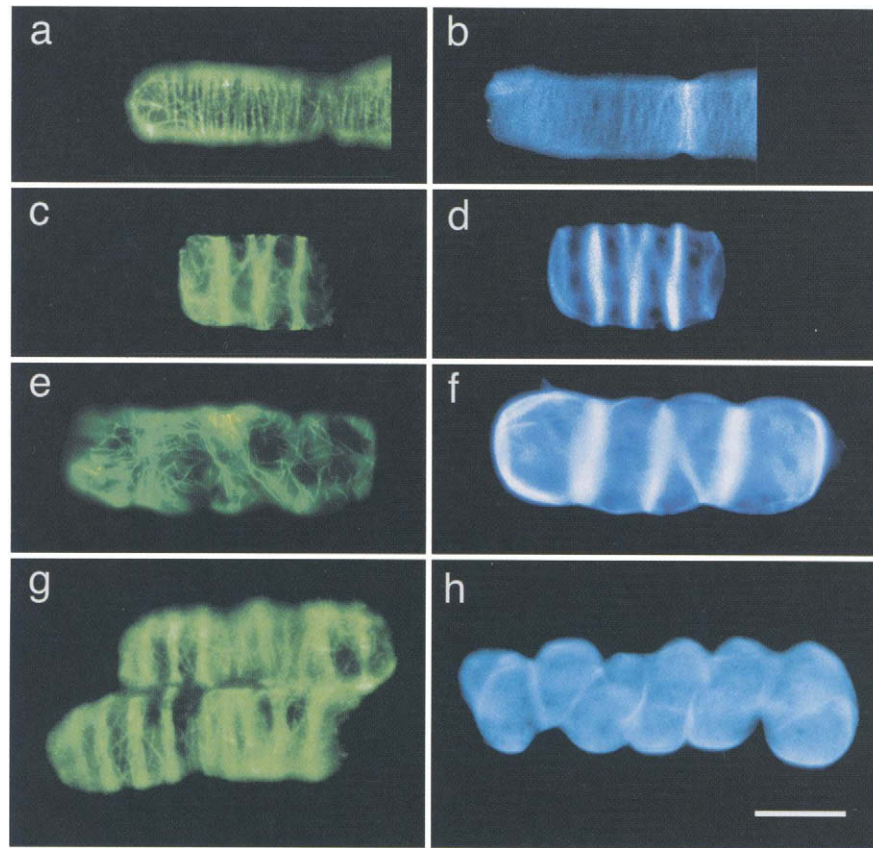


FIGURE 2-41 Mesophyll cells in a wheat (*Triticum aestivum* L.) leaf. The cells show ballooning separated by constrictions. Constrictions are formed by the localized deposition of wall material in which MTs provide the scaffolding. MTs are shown by immunofluorescence in a,c,e, and g. The new wall material is shown by calcofluor staining of new cellulose in corresponding photographs b,d,e, and f. Subsequent to constrictions being formed, the intervening parts grow and balloon out. Bar: $\sim 20\mu\text{m}$. From Jung and Wernicke (1990) with permission.

Vessel elements are unique examples of differential expansion. In some angiosperms, they expand enormously over their precursor cell before they lay down a secondary wall. In roots of maize (*Zea mays*), for instance, the precursor procambial cells are about $20\mu\text{m}$ in diameter and about $200\mu\text{m}$ long; a differentiated metaxylem vessel element, by comparison, is about $200\mu\text{m}$ in diameter and about $2000\mu\text{m}$ long. The differentiating vessel elements first expand laterally and then elongate—enlargement in the two directions is kept temporally separate (Fig. 2-42). In secondary xylem of angiosperms, the vessel elements do not elongate significantly over their precursor derivatives from the cambium, but expand radially. In some ring-porous woods, such as ash (*Fraxinus americana*) and black locust (*Robinia pseudo-acacia*), the individual vessel elements expand radially several hundredfold over their precursor cells. These enormous enlargements

involve single cells in longitudinal files, but they are accommodated by the neighboring cells. The expansion probably involves an uptake of water following a rise in osmotic pressure of the cell, but to the author's knowledge, no measurements of osmotic pressure in these cells have been made. The primary wall is very thin, and it is unclear what controls the directionality of expansion. It could be that the directionality is controlled by ordered arrays of cortical MTs and plasma membrane.

It is only after the cell expansion is complete that a secondary wall is laid down. The secondary wall is deposited in precise patterns inside the primary wall, and these patterns are defined by the cortical MTs (Fig. 2-43). The secondary wall is deposited only in those places that are demarcated by bands of cortical MTs. After the secondary wall has been laid down the MTs disappear.

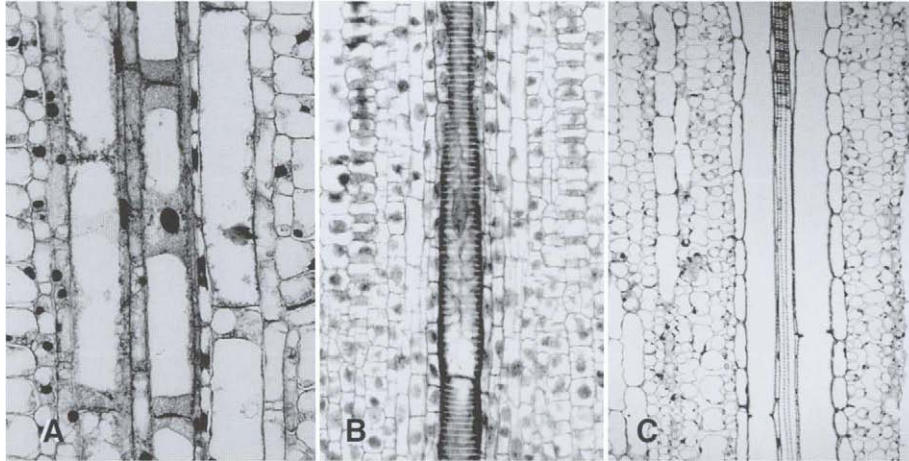


FIGURE 2-42 Elongation and radial growth of differentiating metaxylem vessel elements in maize (*Zea mays*). The vessel elements originate from procambial cells and undergo radial expansion and elongation prior to laying down a secondary wall. In A, the differentiating elements are near the end of the cell expansion phase. In B, the cells have deposited a secondary wall which is also lignified, but the nucleus, cytoplasm, and end walls are still intact. In C, complete autolysis has occurred, and remnants of the end walls appear as pegs from side walls. Some idea of the size of the original procambial cells can be gained from the size of the cells adjacent to the vessel elements seen in B.

4. MICROTUBULES ARE DYNAMICALLY UNSTABLE ENTITIES

As part of the cytoskeleton, MTs play several very important roles. They are involved in delineating the

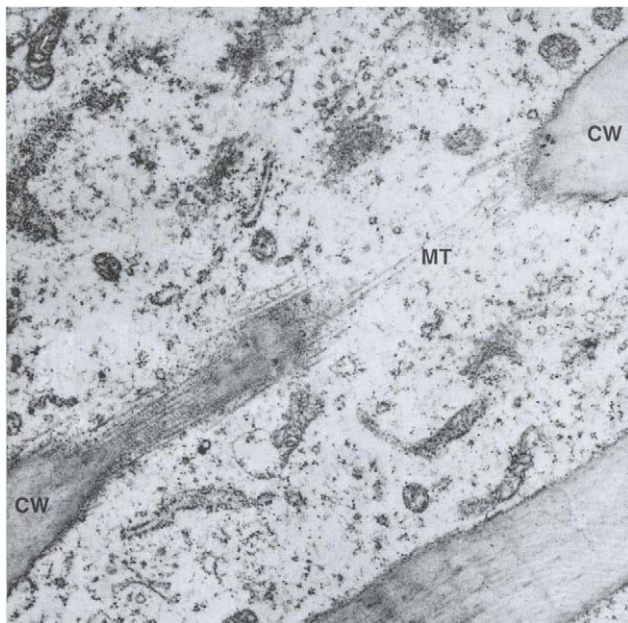


FIGURE 2-43 Parallelism between cortical microtubules (MT) and secondary wall (CW) deposition in differentiating tracheary cells of maize. Part of the secondary wall is cut glancingly exposing the underlying cortical cytoplasm and microtubules. Courtesy of A. P. Singh.

future plane of cell division, in forming the mitotic spindle apparatus, in formation of the phragmoplast, in anchoring the interphase nucleus, in determining the orientation of new cellulose fibrils in walls, and in prepatternning the deposition of the secondary wall in tracheary cells in xylem. Commensurate with these multiple roles, MTs show a remarkable dynamism, they disappear in one location and reappear in another, and it has been suggested that MTs exist in a state of dynamic instability. New techniques that allow visualization of MTs in living cells, using fluorescently labeled heterologous tubulin from brain cells of cows or pigs, coupled with CLSM, have confirmed that MTs are depolymerized in one location and repolymerized in another. There is a pool of tubulin monomers in the cell that is drawn upon for the assembly of MTs in the desired location. The assembly, in turn, is regulated by a variety of other proteins, concentration of $[Ca^{2+}]$, and, for PPB and cortical arrays, possible asymmetries in the PM, which serve as nucleation centers.

Environmental factors such as red or blue light are known to affect patterns of growth. For instance, fern protonemal cells given red light elongate longitudinally without cell divisions. Growth occurs at the tip of the cell, and the subapical region of the filament just below the tip shows transversely arranged cortical MTs and cellulose microfibrils (Fig. 2-44). Further down the length of the filament, both cortical MTs and microfibrils show an arrangement parallel to the long axis of the cell. If the protonemal cells are given blue light, after a short lag period, the tip swells and the transverse arrangement of cortical MTs and microfibrils in the

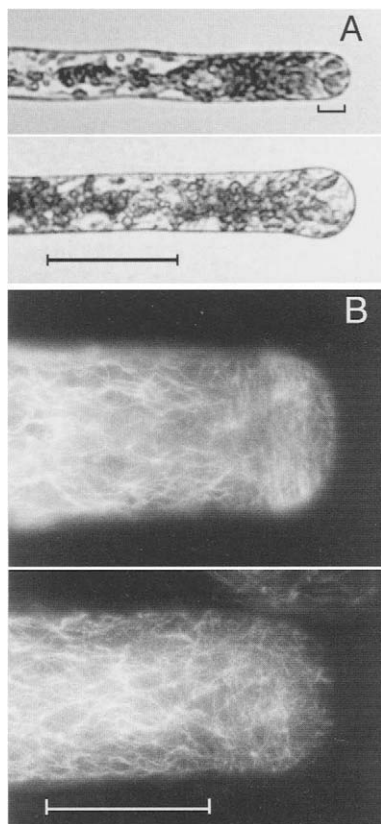


FIGURE 2-44 Protonemal cells of a fern (*Adiantum capillus-veneris*) grown under red or blue light. (A) Protonemal cells grown under red light (top) or irradiated with blue light for 4 h (bottom). The bracket in the top Figure indicates the subapical region. Bar: 50 μ m. (B) Immunofluorescence micrographs of the MT arrangement. Protonemal cells cultured under red light (top) were irradiated with blue light for 30 min or 3 h (bottom). The transverse arrangement of MTs seen in the subapical region of cells cultured under red light is not found in the subapical region in cells irradiated with blue light. Bar: 20 μ m. From Murata and Wada (1989).

subapical region is lost. This is followed a few hours later by cell divisions, which change the growth pattern from one-dimensional filamentous growth to two-dimensional growth in a flat plane. In dicot seedlings, light affects a range of morphogenetic changes, including stem growth, cotyledonary expansion, and greening, when seedlings that are grown in dark are brought to light. How the light signal is perceived in some of these cases and brings about morphogenic changes is covered in Chapters 26 and 27.

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SECTION I. CELL WALLS

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Embryogenesis

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1. INTRODUCTION

Chapter 1 showed that the body plan of the embryo, i.e., the apical-basal polarity and the demarcation of

the embryonic organs and tissue layers, is laid down during early embryogenesis. Embryonic organs consist of the root and shoot apices, the intervening root-shoot axis, and cotyledons (one in monocots and two in dicots). Embryonic tissue layers are the protoderm (the precursor of epidermis), the ground meristem, and the procambial tissue. This body plan is carried into the seedling and the adult plant, but, as mentioned earlier, most of the adult plant body and tissues are formed postembryonically by the activities of the root and shoot apical meristems and lateral meristems such as vascular cambium.

Embryo development has been studied in many plants, and detailed accounts are available in several excellent texts [e.g., Johri *et al* (1992)]. The emphasis in recent years has been on an elucidation of pattern formation during embryogenesis, i.e., how the different embryonic organs and tissues layers come to be established from a single cell, the zygote, using molecular genetic techniques. These techniques are central to much of the information presented in this book. They are outlined in Appendix 1 at the end of Section I in this book. Those unfamiliar with them should read the Appendix 1 before proceeding further. The information on patterning gained from the genetic approach is correlated with the analysis of cell lineages. Because plant cells do not move after division and their patterns of origin are preserved, cell lineages in plants are commonly deduced from an analysis of cell boundaries in tissue sections. More rigorous analyses using cell-specific markers and division patterns, i.e., clonal analysis, are still very few (e.g., *Arabidopsis* root development). Only a few flowering plants have been studied using

genetic techniques, notably *Arabidopsis*, maize, and rice, and a few general patterns are beginning to emerge; it must be recognized, however, that there will be variations as more plants are examined. Other approaches include experimental manipulation of zygotic embryos, including *in vitro* fertilization, and somatic embryogenesis.

This chapter deals with embryo development in *Arabidopsis* and some supporting evidence from maize and rice, followed by possible factors that may influence patterning in plant embryos. A section on somatic embryogenesis concludes the chapter.

2. EMBRYOGENESIS IN *ARABIDOPSIS*

Figure 3-1 illustrates the development of the *Arabidopsis* embryo. The zygote enlarges and elongates before the first asymmetric division setting out the apical cell, which gives rise to the embryo proper, and the basal cell, which gives rise to the suspensor. The apical cell divides twice vertically and then transversely to form an eight-celled proembryo (the octant stage) in which an upper tier of four cells is set apart

from a lower tier of four cells. The basal cell divides transversely several times to give rise to a suspensor of eight to nine cells, the uppermost of which, the hypophysis, forms the third tier and participates in embryo formation. These three tiers have been referred to as apical, central, and basal tiers, respectively, and the apical-basal body plan of the future embryo/seedling is traceable to these tiers. Subsequent cell divisions in the octant embryo result in the formation of the globular embryo. Later localized divisions and growth, at the pole away from the suspensor, lead to the formation of two cotyledons, whereas elongation of the central region leads to the formation of the shoot-root or apical-basal axis. A root apical meristem is distinguishable at the late heart stage, and early in the torpedo stage a shoot apical meristem can be distinguished between the two cotyledons.

The major tissue layers are also laid down very early. Already at the octant stage of the proembryo, periclinal divisions set apart the protoderm from the central cells. Cell divisions continue in the central cells, with the protoderm keeping pace with anticlinal divisions, and subsequent periclinal divisions set aside the ground meristem from the innermost cells, the procambial cells.

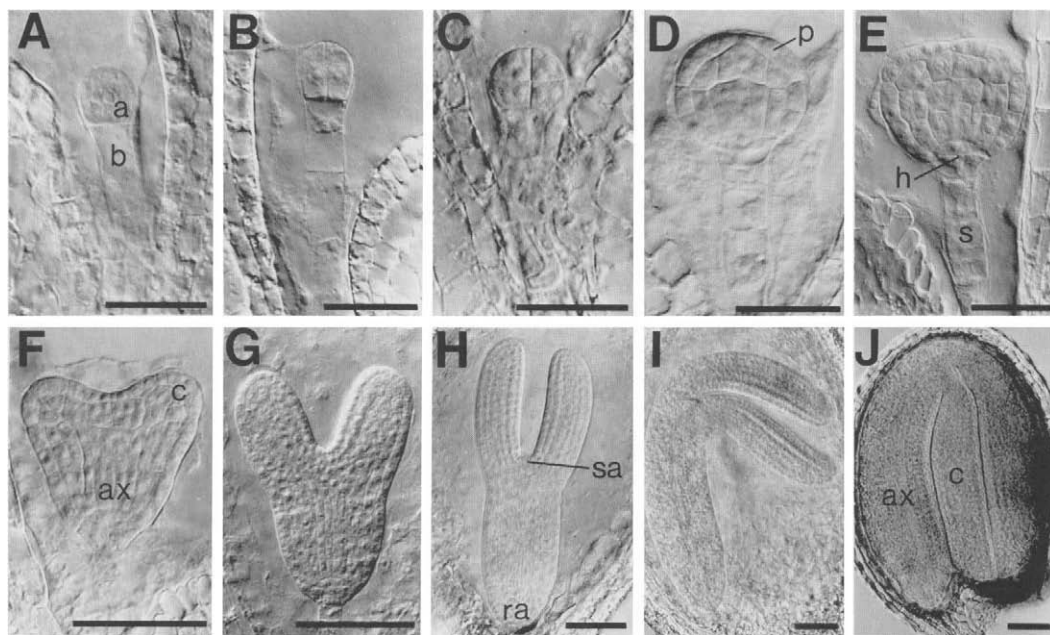


FIGURE 3-1 Embryo development in *Arabidopsis*. (A) Apical and basal cells after the first division of the zygote. (B) Two- or four-celled embryo proper. (C) Octant stage embryo. (D) Early globular stage and (E) transition stage embryo. (F) Early heart stage and (G) late heart stage embryo. (H) Linear cotyledon and (I) curled cotyledon stage embryo. (J) Mature embryo. a, apical cell; ax, axis; b, basal cell; c, cotyledons; h, hypophysis; p, protoderm; ra, root apex; s, suspensor; sa, shoot apex. Bars A–E: 25 μ m; F–J: 50 μ m. From West and Harada (1993).

Thus, the body plan of the embryo, including the demarcation of the major organs and the major tissue zones, is nearly complete by the late heart or early torpedo stage. In chronological terms, this series of events represents only the first quarter of embryogenesis; later steps involve an increase in size, deposition of storage food materials, and maturation, desiccation, and quiescence (see Chapter 18).

3. GENETIC DISSECTION OF PATTERN FORMATION IN *ARABIDOPSIS* EMBRYOS

A study of mutants coupled with deduced cell lineages from tissue sections indicates that the embryo development in *Arabidopsis* involves a superposition of two semi-independent patterns: an apical-basal pattern and a radial pattern.

3.1. Apical-Basal Pattern

Mutant embryos/seedlings in *Arabidopsis* that are well characterized fall into four major phenotypes. All are single gene mutations with several alleles. The extreme forms of the four types are represented by (i) *gurke*, where the entire apical region, consisting of the shoot apex, the cotyledons, and the upper hypocotyl, is missing; (ii) *fackel*, in which the central region consisting of the rest of the hypocotyl and upper part of root is absent; (iii) *monopteros*, where both the central region and the basal region, consisting of most of the root and root apex, are missing; and (iv) *gnom* (also called *emb30*), in which both the apical and basal regions are lost (Fig. 3-2).

The *gnom* embryos appear as more or less spherical blobs without any apical-basal polarity and are thought to result from the deletion of apical and basal regions. In other phenotypes, the loss of one or even two segments does not affect the development of an adjacent neighbor; for instance, in *gurke*, the central and basal regions are unaffected; in *monopteros* embryos, the central and basal regions are missing, but the apical region is normal; and in *fackel* embryos, the cotyledons appear connected directly to the root.

These mutants can be recognized by heart stage (e.g., *gnom* embryos), or even earlier in globular stage (e.g., *monopteros* and *fackel* embryos), which suggests that the embryonic domains are set up early, that is, the normal, or wild type genes regulating these domains act early in embryogenesis, such that mutations in them affect the future development of a whole sector or domain of the embryo.

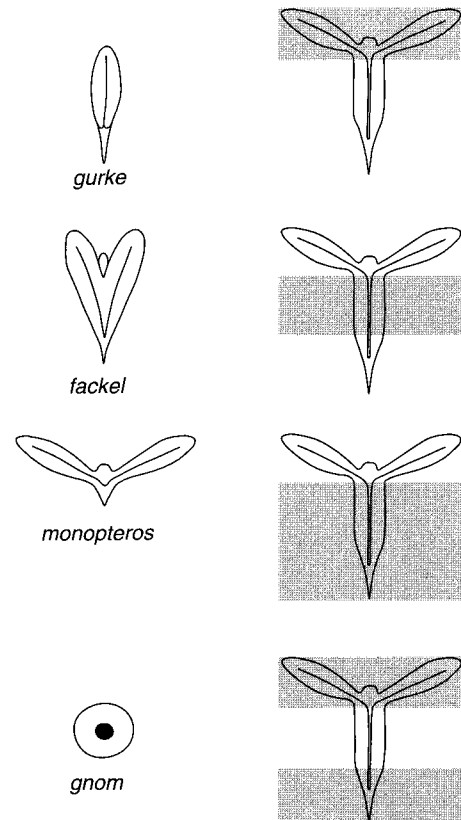


FIGURE 3-2 Mutant embryos from *Arabidopsis*. Shaded areas represent embryo parts that are missing. From Mayer *et al.* (1991) with permission.

A study of cellular patterns in tissue sections provides information about cell lineages and how each sector is derived. Such a study indicates that the apical region of the embryo, consisting of the shoot apex and most of the cotyledons, is derived from the apical tier of the proembryo; the central region, consisting of the hypocotyl and part of embryonic root and root apical meristem, comes from the central tier, and the basal region, consisting of the remainder of the root meristem – the quiescent center and the initials of root cap, comes from the basal tier in the proembryo (Fig. 3-3).

The derivation of the quiescent center and root cap initials from the basal tier is confirmed by other mutants. The *hobbit* (*hbt*) mutant has cotyledons, a hypocotyl, and a rudimentary root, but lacks a defined root meristem including the quiescent center and columella root cap initials. The defect is traceable to aberrant divisions or a lack of divisions in the hypophyseal progenitor of the basal tier. The *bodenlos* mutant also lacks a hypophysis and, hence, does not form the primary embryonic root; secondary roots are formed normally, however.

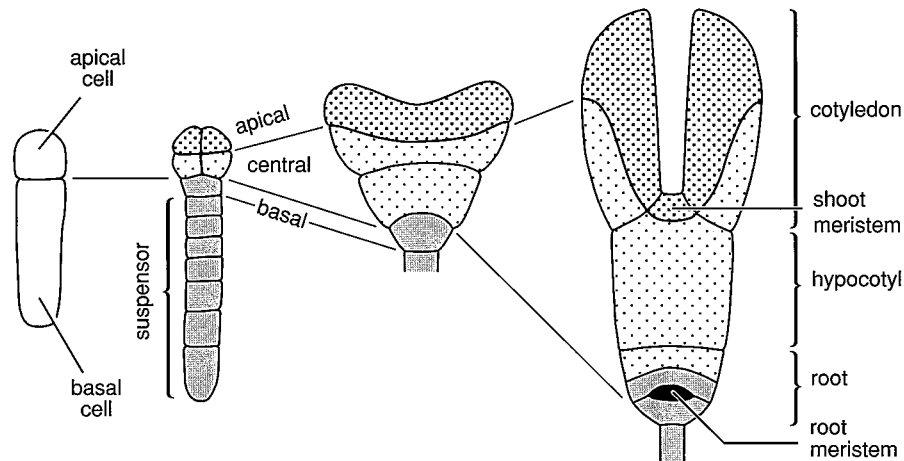


FIGURE 3-3 Possible derivation of various sectors in the *Arabidopsis* embryo. The apical tier is thought to give rise to the shoot apex and most of the cotyledons; the central tier to part of the cotyledons, the hypocotyl, and upper parts of the root; and the basal tier to the remainder of the root, including the root meristem. The sectors are more or less autonomous in their development. Adapted from Jürgens (1995) with permission from Elsevier Science.

3.2. Radial Pattern

An analysis of division patterns indicates that at the octant stage of the proembryo, periclinal divisions set apart the protoderm from the central cells, and later, in the globular/heart stage, the ground meristem from innermost cells, the procambial cells. Further demarcation of tissue layers seems to occur similarly along the apical-basal axis, but gives rise to tissue layers whose eventual fates are different in the future root or shoot regions. In the region destined to form the root, the ground meristem cells divide periclinally to separate an outer layer which forms the future cortex, and an inner layer which differentiates as the endodermis. The procambial cells also divide periclinally to set aside an outer layer that becomes the future pericycle, and an inner layer that forms the vascular tissues (Fig. 3-4). In the region destined to form the shoot, the inner layer (after the periclinal division in the ground meristem cells) differentiates as starch-sheath cells; and a pericycle is not clearly defined. The differentiation of xylem and phloem tissues in the procambial cells also occurs along different lines in the root and shoot regions (see Chapter 1).

Several radial patterning mutants where one or another primary tissue is missing or modified are known. Thus, the periclinal division separating the protoderm layer from the central cells does not occur in the *knoll* mutant. Periclinal divisions in the ground meristem layer result in separation of the cortex from endodermis (or starch-sheath cells). In

mutants, such as *short root* (*shr*) and *scarecrow* (*scr*) this separation is not affected. In other mutants, the organization of vascular tissues and pericycle (e.g., *gollum*) or that of vascular tissues is altered. In *wooden leg* (*wol*) mutant, for instance, fewer than normal number of vascular cells are formed in the hypocotyl and root and phloem tissue fails to be specified. In still other mutants, more than normal cell divisions occur and lead to an excessive number of cells in all cell layers; nonetheless, the radial pattern of tissue types remains undisrupted (e.g., *fass* and *tonneau*).

Double mutant analysis helps in further clarification of the roles of some of these mutant loci in roots. A *shr fass* double mutant shows additional cell layers but no endodermis specification; hence, *SHR* is thought to specify endodermis. The *scr fass* double mutant, in contrast, has a single endodermal layer surrounded by multiple layers lacking endodermal attributes. Hence, *SCR* is believed to be involved in controlling the periclinal division that separates the cortical and endodermal cell layers. A *wol fass* double mutation restores phloem tissue; indicating that *WOL* primarily regulates cell division and secondarily phloem specification.

In still another group of mutants, the *pilz* (mushroom-like) group, the embryo remains small consisting of only one or a few large cells and the endosperm does not cellularize. Apparently, the microtubule assembly or organization is defective leading to failure of proper mitotic division and cytokinesis, though cell cycle events progress normally.

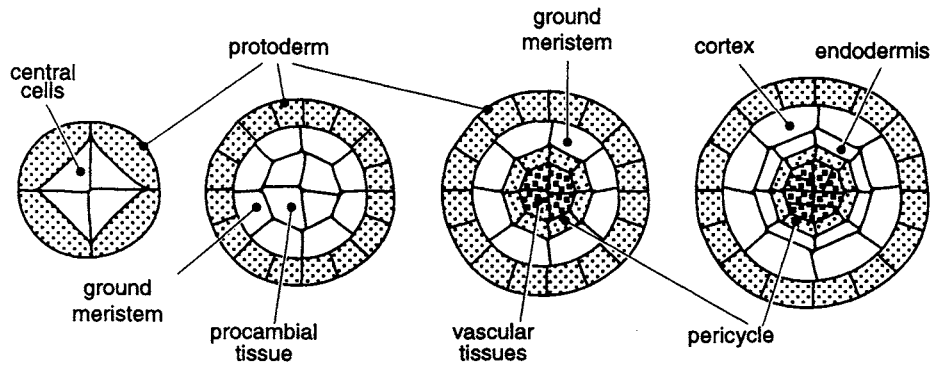


FIGURE 3-4 Delineation of the radial pattern in *Arabidopsis* embryo. Periclinal divisions in the eight-celled embryo separate central cells from protoderm. Subsequently, central cells give rise to the ground meristem and procambial tissue. In roots, the procambial tissue gives rise to vascular tissues and pericycle, whereas the ground meristem gives rise to the cortex and endodermis. Adapted from Jürgens (1995) with permission from Elsevier Science.

3.3. Other Partitioning Events

At late globular stage, the apical domain of the embryo is partitioned into a central area which becomes the shoot apical meristem, and the surrounding area from which cotyledons develop as localized outgrowths. Once originated, the shoot meristem and cotyledons follow different and, unless perturbed, independent fates. The shoot meristem maintains an identity that constrains the component cells to proliferate, but not to differentiate into mature organs/tissues. The cotyledons, by contrast, become determinate organs and may be considered as embryonic leaves. That shoot meristem, once defined, is distinct from cotyledons is supported by mutant analysis. Mutants that define the shoot meristem or regulate its size are genetically distinct from those that affect the cotyledons. For instance, the *Arabidopsis* mutant *shoot meristemless* (*stm*), in a homozygous state, fails to form a shoot meristem in the embryo. In *zwille/pinhead* a rudimentary shoot meristem is formed, but fails to establish itself and adventitious shoot meristems are formed postembryonically in the axils of the cotyledons. In some others, fewer cell divisions occur in the central zone and a smaller than normal shoot meristem results (e.g., *wuchsel*). In still others, more than normal cell divisions occur and a larger shoot meristem results (e.g., *clavata*). These mutations affect only the shoot meristem, not the cotyledons. By contrast, mutations that affect cotyledons or lateral organs (e.g., *aintegumenta*, *leafy cotyledon*) affect cotyledons and/or other lateral organs, not the shoot meristem.

In the root, the protoderm layer is partitioned into the epidermal layer and lateral root cap. The cells in the epidermis are again partitioned into cells that de-

velop root hairs (trichoblasts) and those that do not (atrachoblasts). In mutants such as *glabra2* (*gl2*) this distinction between epidermal cells is lost and all cells behave as trichoblasts. Many other mutants affecting root hair and/or trichome development in leaves and stems are known.

In summary, an analysis of mutant phenotypes and of deduced cell division patterns in *Arabidopsis* indicates that embryo development proceeds in a step wise blocking out of sectors, or domains, which in their subsequent development are more or less independent of each other. The young embryo is partitioned into a root pole and a shoot pole, and the shoot pole into the shoot meristem and cotyledons. Axialization of the region between the root and shoot poles gives rise to the hypocotyl and part of root. Later, as primordia of lateral organs arise at the shoot meristem on a continuous basis, they also follow separate destinies, such that mutations in lateral organs do not affect the shoot meristem, or *vice versa*. The domains in the young embryo are specified by the operations of two separate sets of genes, which regulate, on the one hand, an apical-basal pattern which sets out the major embryonic organs, and, on the other, a radial pattern which sets out the major primary tissues. These patterns and the resultant organs/tissues are summarized in Fig. 3-5. That the two patterns operate more or less independently is supported by the fact that the *gurke*, *monopteros*, and *gnom* mutants may show separation of a protoderm from the central cells; even a procambial region may be distinguished in these mutant embryos. Nonetheless, there is some interaction between the two patterns as well. For instance, the expression of *SHOOT MERISTEMLESS* gene (see Section 5 below) in *Arabidopsis* embryo is dependent on prior radial patterning in the apical portion of the embryo.

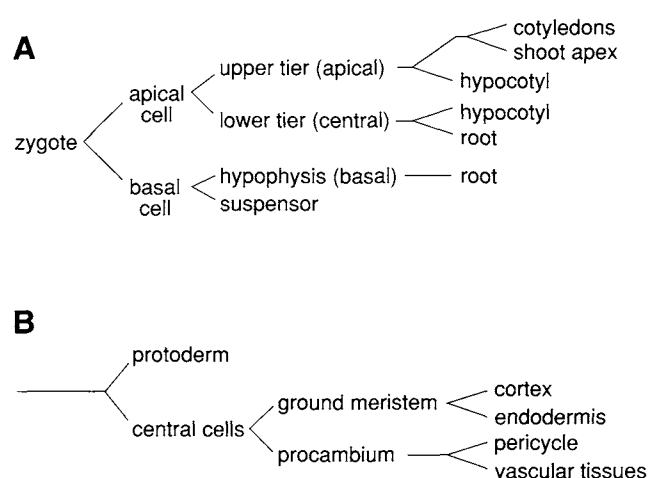


FIGURE 3-5 Stepwise blocking out of domains (sectors) in embryo development. (A) Apical-Basal pattern setting out the major embryonic organs. (B) Radial pattern setting out the primary tissues layers.

4. ANALYSIS OF MUTANT PHENOTYPES IN MAIZE AND RICE

Analyses of mutant embryos in maize and rice, both monocots, have yielded similar results. Mutant embryos are described which remain as globular-(or club-shaped) embryos and do not form root or shoot poles (e.g., in rice, *gle1, gle2; cle1-1, cle1-2*). The *gle1, gle2* remain spherical, ca. 300 μm in diameter, whereas the *cle1-1, 1-2* embryos enlarge and become oblong, about 500–800 μm in the long axis. As in *Arabidopsis*, the *club-shaped* mutants in rice may show procambial differentiation. Other mutants in maize and rice form roots but no shoots [e.g., in rice *shootless (shl1, shl2)*], and in still others, the shoot apex may be displaced from the normal position. Patterning in these embryos also seems to proceed along an apical-basal axis for major organs and radially for major tissues.

5. CLONING AND CHARACTERIZATION OF GENES

Many genes identified from analysis of embryo mutants in *Arabidopsis* have been cloned and characterized (Table 3-1; for methodologies of cloning genes from mutants, see Appendix 1). Pattern formation in plants, where cells are fixed in space, involves at least two phenomena: (i) oriented cell divisions and cell growth, and (ii) determination of fate, or cell-and

tissue-specification (for a discussion of these topics, see Chapter 4). It is not surprising, therefore, that many of the cloned genes are reported or are suspected to play a role in cell divisions/cell growth (e.g., *FACKEL, KNOLLE, SCARECROW, AINTEGUMENTA*), while some others are involved in specification of organ- and/or tissue-fate (e.g., *KNOTTED 1, SHOOT MERISTEMLESS, WUCHSEL, L1, GL2*). Genes such as *GNOM* seem to regulate the acquisition of apical-basal polarity in the embryo, whereas the *MONOPTEROS* gene regulates axialization and vascular patterning in *Arabidopsis* embryo and adult plant; they encode proteins thought to be involved in auxin transport and/or action and are discussed further in Chapter 14.

Transcription factors often regulate the activities of whole sets of structural genes, and prescribe broad sectors of tissue-and/or organ-fate. They belong to many different classes (see Appendix 1). Genes known to be encoding transcription factors include, *MONOPTEROS* which encodes a transcription factor of the $\beta\alpha\alpha$ type, *SCARECROW* which encodes a transcription factor of the GRAS type, *AINTEGUMENTA* which encodes a transcription factor of the AP2-domain type, and the genes, *KNOTTED, SHOOT MERISTEMLESS, WUCHSEL, L1*, and *GL2* which encode homeodomain-containing transcription factors. Still other genes seem to be involved in perception of environmental and/or intercellular signals. For example, *CLAVATA1* and *WOL* encode two different types of receptor-like proteins; *CLAVATA1* encodes a receptor kinase, while *WOL* encodes a modified two-component signaling protein (for two-component signaling and receptor kinases, see Chapters 21 and 25).

In summary, molecular genetic studies on plant embryogenesis, though relatively few and confined to a few species, suggest that embryo development proceeds via two separate sets of pattern specifications, an apical-basal pattern which delineates the major organs and a radial pattern which specifies the major primary tissues. The two pattern specifications seem to proceed more or less independently; nonetheless, there is some interaction between the two patterns. Many regulatory genes encoding transcription factors, or proteins involved in signaling, or in auxin transport or action, participate in axial or radial pattern formation. Some transcription factors specify organs/tissue layers/cell types, others regulate events associated with cell division and/or growth. There appear to be hierarchies of genes that become activated at different times in embryo development, and which specify blocs, sub-blocs, and sub-sub-blocs, in descending order in shoot or root development, but more genes including target structural genes need to be identified before definitive conclusions can be reached.

TABLE 3-1 Cloned Genes That Act Relatively Early in Embryogenesis and Their Known or Postulated Sites of Action^a

Gene	Site of action	Nature of encoded protein	Reference
<i>GNOM</i>	Apical-basal polarity	Involved in vesicular traffic	Shevell <i>et al.</i> (1994)
<i>MONOPTEROS</i>	Axialization and vascular development	Transcription factor ($\beta\alpha\alpha$ type)	Hardtke and Berleth (1998)
<i>FAKEL</i>	Membranes, affects cell division/growth	A C-14 reductase involved in sterol synthesis	Schrick <i>et al.</i> (2000)
<i>KNOLLE</i>	Protoderm separation	Syntaxin-like protein (cell plate formation)	Lukovitz <i>et al.</i> (1996)
<i>SCARECROW</i>	Separation of cortex and endodermis or starch-sheath cells	Transcription factor (GRAS type)	Di Lorenzo <i>et al.</i> (1996), Wysocka-Diller <i>et al.</i> (2000)
<i>KNOTTED 1</i>	Specification of shoot meristem	Transcription factor (HD type)	Vollbrecht <i>et al.</i> (1991)
<i>SHOOT MERISTEMLESS</i>	Specification of shoot meristem	Transcription factor (HD type)	Long <i>et al.</i> (1996)
<i>WUCHSEL</i>	Maintenance of shoot meristem	Transcription factor (HD type)	Laux <i>et al.</i> (1996)
<i>CLAVATA 1</i>	Regulation of shoot meristem size	Receptor-like protein	Clark <i>et al.</i> (1997)
<i>AINTEGUMENTA</i>	Regulation of size of lateral organs	Transcription factor (AP2-domain type)	Mizukami and Fischer (2000)
<i>L1</i>	Specification of tunica 1 layer	Transcription factor (HD type)	Lu <i>et al.</i> (1996)
<i>GL2</i>	Specification of atrichoblasts in root epidermis	Transcription factor (HD type)	Di Cristina <i>et al.</i> (1996)
<i>WOL</i>	Specification of vascular tissues	Receptor-like protein	Mahonen <i>et al.</i> (2000)

^aAll are from *Arabidopsis*, except *KNOTTED 1*, which is from *Zea mays*.

6. EMBRYOGENESIS IN OTHER PLANTS

Although some conclusions regarding embryo development have emerged from a study of *Arabidopsis*, it must not be assumed that all plant embryos follow the same pattern. Two important qualifications must be noted. In *Arabidopsis* (or *Capsella*, both members of Brassicaceae), cell divisions during embryogenesis are regular and cell lineages are easily followed. This is not necessarily true for all plants. There are wide variations in developmental patterns among angiosperms, let alone other groups of vascular plants, though for any single group there is a norm. For example, in about 10% of dicots the first division of the zygote is oblique to longitudinal, not transverse. In plants, such as cotton (*Gossypium hirsutum*), the early divisions are quite irregular and there are no precise cell lineages, yet later globular and heart-shaped embryos are formed. Thus, apical-basal polarity, or even axialization (morphological directionality without polar asymmetry), may not occur until much later. Secondly, even though there is a normal pattern for a plant (or group), the phenomenon of open differentiation referred to in Chapter 1 allows deviations from the norm, should environmental conditions change. There are many examples of major deviations from normal development when embryos are

exposed to chemicals, including hormones or inhibitors of hormone action or transport (see Chapter 14).

7. CONTROL OF PATTERNING IN EMBRYO DEVELOPMENT

Embryo development in animals is usually described in terms of an anterior-posterior axis and a dorsoventral axis. Different mechanisms are used in different animal groups to establish these axes, but in *Drosophila*, both the axes are partly determined by maternal genes, and the anterior-posterior axis of the embryo is already visible in the unfertilized egg (Gurdon, 1992). This has led to two questions regarding embryo development in plants: (1) What is the basis for cytoplasmic asymmetry in the fertilized zygote—is it a carryover from the asymmetry in the egg cell, which in turn is affected by maternal genes, and (2) do maternal genes regulate pattern formation in embryo development?

7.1. The Zygote Can Set Up Its Own Polarity

Several light and electron microscopic studies have shown that the cytoplasmic organization of the zygote is asymmetric before the first transverse division, with

more endoplasmic reticulum (ER) and organelles, such as mitochondria and plastids, concentrated toward the apical (or chalazal) end and one or two large vacuoles toward the basal (micropylar) end. The unfertilized egg cell shows a similar cytoplasmic asymmetry (Fig. 3-6), and it is likely that *in situ* (in place, within the plant) the egg cell, or maternal influences, determines the plane of the first asymmetric division of the zygote.

Nonetheless, several instances indicate that the zygote can set up its own polarity and that new asymmetries can be established in an otherwise radially symmetrical cell. This section reviews some evidence from the *in vitro* fertilization of male and female gametes, somatic embryogenesis, and apomixis.

i. *In vitro* fertilization. A determination of the causal factors for the establishment of polarity in embryos of seed plants *in planta* is not possible because embryo development occurs while it is surrounded by other tissues, e.g., female gametophyte in gymnosperms or endosperm in angiosperms, and, in both taxa, by still another maternal tissue, i.e., the seed coat. However, recent improvements in microdissection and micro-manipulation techniques have made it possible to isolate the embryo sac and egg cell from ovules and also

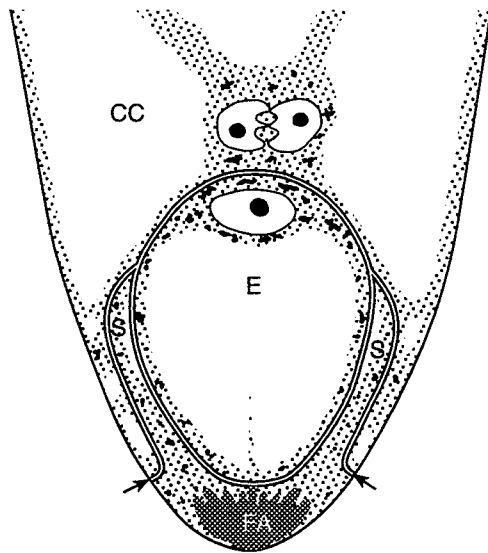


FIGURE 3-6 Schematic drawing of a female germ unit in an angiosperm. The female germ unit consists of the egg cell (E), central cell (CC) with two polar nuclei, two synergids (S), and a filiform apparatus (FA), the site of entry of the pollen tube. The egg cell shows a polar organization with a large vacuole toward the micropylar end and nucleus and other organelles toward the chalazal end. The egg cell will unite with a sperm cell to form the zygote and embryo, whereas polar nuclei will unite with the other sperm nucleus to form the primary endosperm nucleus and endosperm. Synergids are attached to the embryo sac wall at points indicated by small arrows. From Russell (1993).

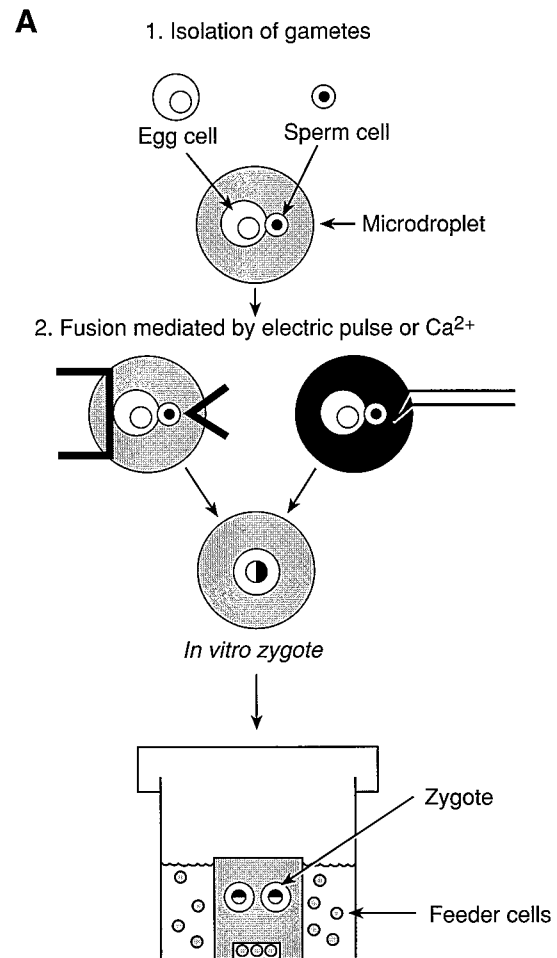


FIGURE 3-7 Technique of *in vitro* fertilization and isolated male and female cells in maize. (A) Protocol of isolation of gametes and fertilization. Ovules at a receptive stage (i.e., when they are ready to receive the pollen tube and male gametes) are harvested and nucellar tissue is softened by cell wall hydrolyzing enzymes. This is followed by a manual dissection of the egg cell, synergids, and the central cell. Male gametes are obtained by bursting pollen grains or pollen tubes in a hypotonic solution. Protoplasts of egg cells and male gametes are brought in contact using glass microcapillaries, and the fusion of membranes is facilitated by short DC electric pulses or by calcium ions. The whole operation is performed on the stage of an inverted microscope. Embryo development from the zygote does not require endosperm or maternal tissues, but is facilitated by "feeder" systems of sporophytically derived nurse cell cultures. Modified from Kranz and Dresselhaus (1996). Courtesy of Erhard Kranz, University of Hamberg. (B) An isolated unit of central cell (cc), synergids (s) and egg cell (e). (C) An isolated sperm and egg protoplast just before fusion.

male gametes, perform *in vitro* fertilization, and generate whole plants from fertilized zygotes (Fig. 3-7). These experiments have been performed so far with maize and are being extended to other plants (e.g., wheat, barley, ryegrass, tobacco, and rape seed). In maize, under the right conditions, the fusion of protoplasts

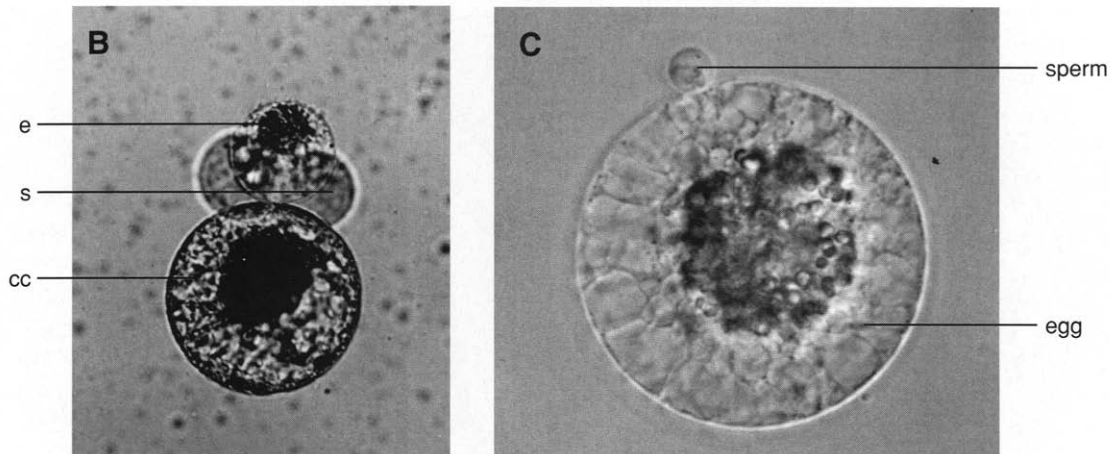


FIGURE 3-7 (continued)

of a male gamete and egg cell occurs very quickly (~ 1 s) and karyogamy follows generally within 60 min, with the first division within ~ 42 – 46 h after fusion. The egg cell may retain some of its polar organization or may lose it before fertilization. Asymmetry of cytoplasm and organelles is reestablished in the zygote before the first division, which is also asymmetric. *In vitro* embryo development copies *in vivo* embryo development; divisions may occur irregularly for a while, but within 12–13 days an oblong embryo with an attached suspensor-like structure is formed. Later, the embryo can be transferred to solid medium and whole plants obtained.

The successful isolation of egg cell and male gametes, and *in vitro* fertilization and zygotic development, allows a dissection of intrinsic and environmental

factors responsible for the polarization and patterning of zygotic embryos in a higher plant. It would also allow an isolation of embryo-specific genes and their precise patterns of expression and roles in development.

Zygotes produced *in planta* can also be isolated and cultured *in vitro*. In this connection, work on egg cells and zygotes of *Pelvetia* and *Fucus* (both members of Fucales among brown algae) is important. Careful and detailed experiments have established that the egg and the newly fertilized zygote are apolar and have a radially symmetrical organization, but environmental cues, such as light and pH, set up gradients that lead to axis formation, which is labile at first but then is stabilized, followed by cytoskeletal reorganization and partitioning of the cell into two cytologically dissimilar halves by the first cell division (see Box 3-1).

BOX 3-1 AXIS FORMATION IN ZYGOTES OF FUCALES

FUCALES ARE COMMON BROWN seaweeds abundant in the upper intertidal shores of temperate oceans. *Fucus* and *Pelvetia* produce relatively large egg cells, up to 1 mm in diameter, in large numbers, which are released into the seawater along with the sperm, and fertilization and embryo development occur in seawater free from maternal influences. The zygotes divide asymmetrically to produce a small rhizoidal cell and a large apical cell. The rhizoidal cell gives rise to the holdfast, which anchors the plant to the rocky substratum, whereas the large cell forms the stipe and fronds. Electron microscopy has revealed that the egg itself and the freshly fertilized zygote are both symmetrical and apolar (Fig. 3-8).

External signals, such as unidirectional light or a pH gradient, provide the cue for the formation of the axis of polarity; this axis is labile for some time (in zygotes of *Fuciales*, up to 10 h postfertilization); then it becomes stabilized or fixed (10–14 h). Among the early signs of axis formation are the flow of an electric current from outside, through the presumptive rhizoid pole, up through the cell, and exiting through the thallus pole; a directed exocytosis of vesicles—“cortical clearing”—at the presumptive rhizoid pole; and a possible involvement of actin filaments in axis formation (cytochalasin B is a drug that disrupts the polymerization of globular actin monomers into actin filaments; a short treatment with cytochalasin B disrupts the axis formation). For axis stabilization, not only are actin filaments necessary, but also

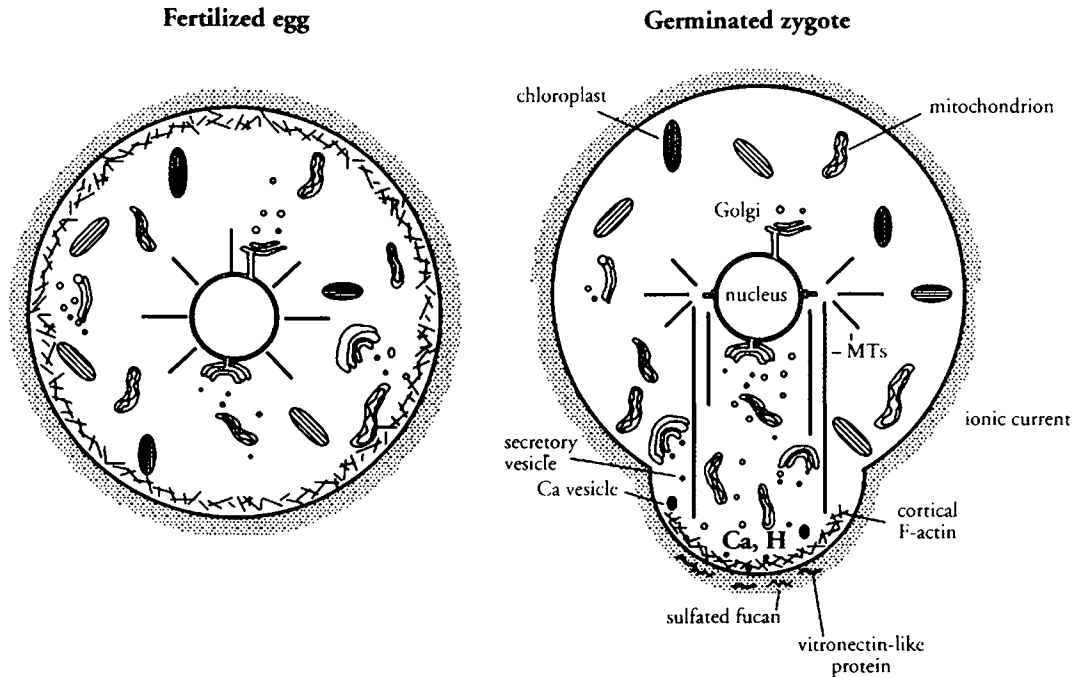


FIGURE 3-8 Axis formation in zygote of *Fucus*. The fertilized egg has no inherent polarity. By the time the zygote germinates, many cellular components, all labeled structures, are unevenly distributed along the polar axis. Adapted from Kropf (1994).

Golgi-derived vesicles carrying cell wall materials need to accumulate at the rhizoid pole. Stabilization of the axis is followed by the asymmetric distribution of zygotic cytoplasm, organelles, and cytoskeleton (14–18 h), which in turn is followed by the first cell division and partitioning of the cell into two cytologically dissimilar halves. Thus, the apical-basal polarity is set before the first asymmetric division.

Some experiments suggest that cues to axis formation (apical-basal polarity) are also imprinted in the cell wall of the zygote. Subsequently, the two cells in the bicelled embryo carry these wall-specific signals, which determine the fates of their progeny. If the walls of the two-celled embryo are enzymatically hydrolyzed and protoplasts are released, the cells dedifferentiate and revert to the zygotic state. If the two cells are separated but are kept within their walls, they follow their own restricted fate. If a protoplast from one cell type is exposed to wall fragments from the other cell, the fate of the former is switched, suggesting that some cell-specific signal is embedded in the wall. Thus, cell walls can carry information for fate determination.

ii. Somatic embryogenesis, or the production of embryos from vegetative or somatic cells, other than zygote, is common in angiosperms as well as several gymnosperms (see Section 8). The carryover of asymmetry from maternal factors in the development of somatic embryos is not likely.

iii. Apomixis, or the production of embryos from cells in the ovule, other than the zygote, is common in several large families of angiosperms. Several different types of apomixis are known, but the concept of the polarity of the egg determining the asymmetry of zygote is difficult to extend to the apomictic embryos that arise from individual cells of nucellus or inner integument (Fig. 3-9).

7.2. Genes That Regulate Pattern Formation during Embryogenesis Are Not Expressed in Maternal Tissues

All genes that have been identified so far, *via* mutant analysis, to play a role in plant embryo development are expressed in the zygote or embryo, not in egg cell or ovular tissues, which indicates that major specifiers of the embryo body plan act after fertilization has occurred (see Table 3-1). In addition to the genes mentioned in Section 5, *TWIN* genes in *Arabidopsis* (see Chapter 4) are thought to suppress the embryogenic potential of the basal cell and its derivatives, which give rise to the suspensor. *GNOM* and

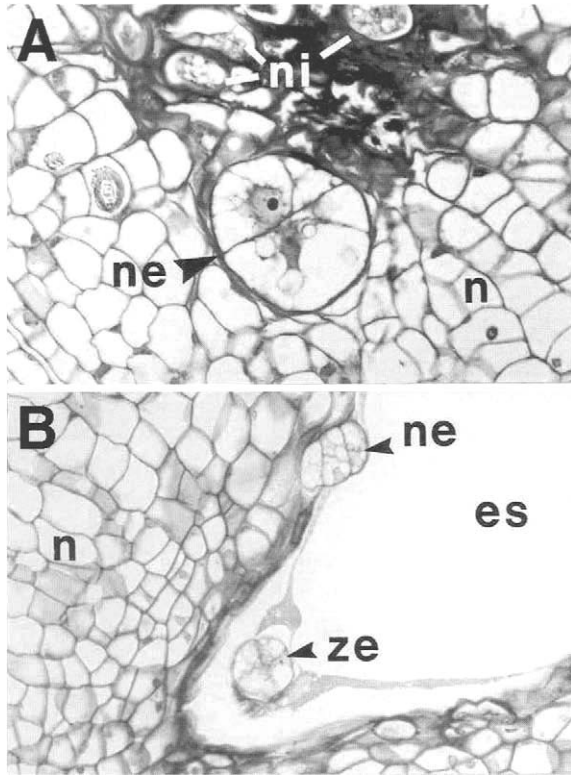


FIGURE 3-9 Production of apomictic embryos from nucellus (n) in *Citrus*. (A) An unfertilized ovule. A nucellar initial cell has divided to produce a two-celled nucellar embryo (ne). Other nucellar initials (ni) can be seen differentiating from the nucellus. (B) A longitudinal section through the micropylar end of a fertilized seed showing the zygotic embryo (ze) and a small nucellar embryo (ne), which is growing into the embryo sac (es). From Koltunow (1993).

TWIN genes are among the earliest acting genes identified thus far. mRNAs of both are expressed only in zygotic or embryonic tissues, not in maternal tissues or in the unfertilized egg.

In summary, while asymmetry of the egg cell may be carried to zygote, and maternal influences may play a role in early embryo development *in planta*, other examples suggest that plant cells can set up their own polarity and give rise to embryos. Moreover, the genes expressed in early embryogenesis are zygotic in nature. Nonetheless, there are reasons to believe that some maternally-inherited alleles keep the embryo and/or endosperm development program suppressed in the female gametophyte up to the time of fertilization. In mutants such as *fertilization-independent endosperm* (*fie*) and *fertilization-independent seed* (*fis*), this suppression is released, and results in a precocious development of endosperm and embryo (from the central cell nucleus and egg, respectively) without double fertilization; even seed and fruit development (from ovular integuments and ovary, respectively) seems to proceed nor-

mally. Usually, however, the embryo and endosperm development is arrested after a certain stage and the seeds atrophy. In the *medea* mutant also, the central cell nucleus proliferates without fertilization leading to a massive development of endosperm. The *FIE* and *MEDEA* genes have been cloned and encode different members of a diverse group of polycomb proteins which in mammals, insects and fungi are known to participate in protein complexes that serve to ensure the stable inheritance of expression patterns through cell division and regulate the control of cell proliferation in developing embryo. It is possible therefore that *FIE* and *MEDEA* proteins participate in similar complexes that keep the endosperm development program silenced in the female gametophyte.

8. SOMATIC EMBRYOGENESIS

As mentioned in Chapter 1, somatic embryogenesis is the production of embryos, and eventually whole plants, from single vegetative or somatic cells. The explant, or source cells, may come from almost any part of the plant body—leaf, stem, root, hypocotyl, and flower—and may include highly specialized cells, such as leaf mesophyll, epidermis, and pollen grains. As a result, the concept has grown that nearly all living plant cells are potentially “totipotent”; totipotent, in this context, means being able to give rise to the whole plant. This statement is subject to some qualifications, however. A functional nucleus is necessary for a cell to be totipotent, which rules out differentiated tracheary cells or sieve elements. A lignified cell wall is also a barrier because lignin makes cell walls rigid; cells can divide, but they cannot grow. Plant cells lack enzymes for the hydrolysis of lignin polymers, and, once deposited, lignin cannot be removed by plant cells (certain bacteria and fungi have the required ligninases to hydrolyze lignin).

Until recently, somatic embryos were difficult to obtain from monocots and conifers. However, improvements in culture techniques, especially more frequent renewal of culture medium, which presumably gets rid of some inhibitory substances released from the cut explants, have made it commonplace to obtain somatic embryos from these groups of plants as well.

8.1. Technique

The explant is taken from a suitable plant part (stem or root or petiole, or leaf discs; usually tissues rich in parenchyma cells, e.g., pith, cortex, mesophyll, or phloem parenchyma from a storage organ, such as carrot

root) and is placed in a culture medium with appropriate nutrients and usually a synthetic auxin, such as 2,4-dichlorophenoxyacetic acid (2,4-D, see Chapter 6). It produces callus, i.e., an unorganized mass of relatively undifferentiated cells, which may have one or more centers of high meristematic activity. These centers, so-called proembryogenic masses (PEMs), are sieved off, a process that also removes the exogenous auxin. PEMs may have hundreds of embryo-like structures, which are thinned out and plated on a solid medium where further development of the embryos continues (Fig. 3-10).

8.2. Induction of Embryogenesis

While the technique for somatic embryogenesis is well developed and almost routine for most sources of explants, the changes that occur at the cell and molecular level prior to and during induction of embryos are still not clearly understood.

Mature and differentiated plant tissues are often polyploid. The haploid DNA content of cells is called the C value. In many differentiating plant cells, nuclei

undergo what is known as endoreduplication, i.e., the DNA content multiplies without an accompanying cell division. Thus, C values of 4C, 8C, 16C, 32C, and even 64C are not unusual in mature cells of leaf epidermis or mesophyll, in phloem or xylem parenchyma cells in storage organs, or in cells of storage tissues, such as seed endosperm, or cotyledons. The reasons for such high ploidy levels are obscure, although they may have something to do with stabilization of the differentiated state. Second, mature and differentiated cells are usually specialized for a function. They may accumulate large amounts of a particular protein or proteins [e.g., mesophyll cells specialized for photosynthesis accumulate large amounts of chlorophyll-binding proteins a and b and ribulose biphosphate carboxylase/oxygenase (RUBISCO), which is the major carbon-fixing enzyme]; may store large amounts of food substances, such as lipids, proteins, and starch, as in seed tissues; or they may be modified in some other ways. Third, differentiated cells show a loss of telomerase activity (see Box 3-2) in some plants, and at least a shortening of telomere length.

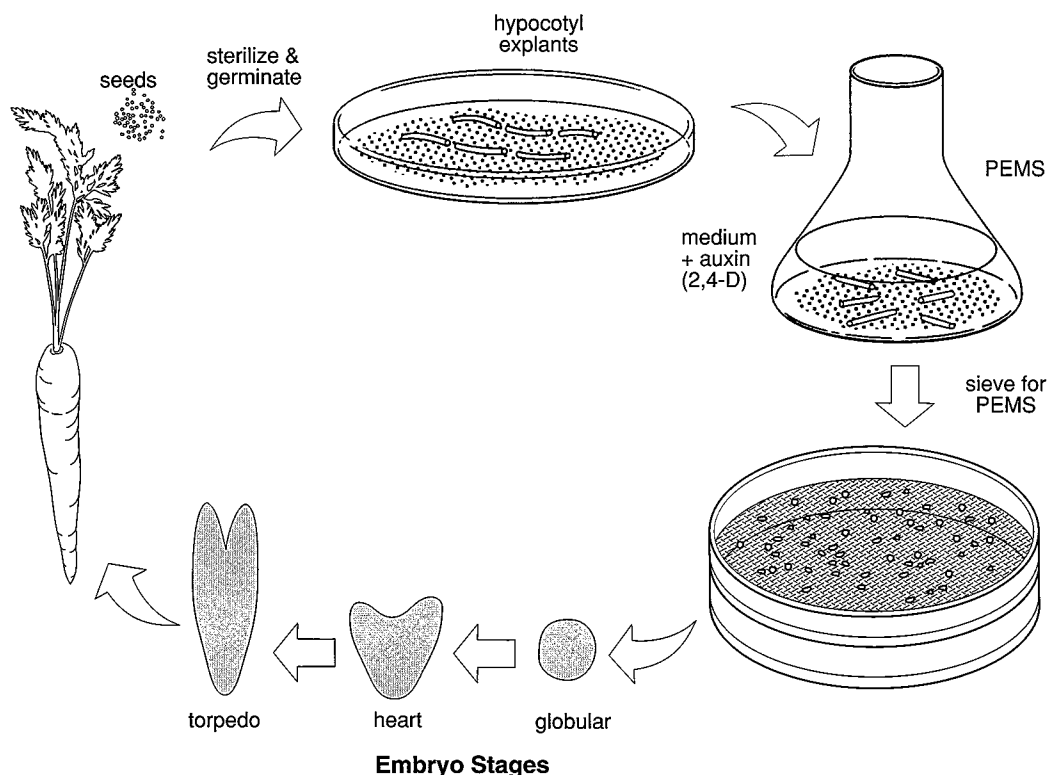


FIGURE 3-10 For production of somatic embryos, explants may be taken from zygotic embryos or seedling tissue (e.g., hypocotyl); mature tissues from older plants, e.g., phloem parenchyma, leaf mesophyll, and even pollen grains, may also be used as a source. The protocol, as developed for carrot hypocotyl explants, shown here, involves (1) production of a callus cell line (or tissue) in the presence of an auxin, 2,4-dichlorophenoxyacetic acid (2,4-D); (2) selection of an embryogenic subpopulation—the “proembryogenic masses” (PEMs)—through sieving or gradient fractionation, which (3) also removes the auxin from the culture medium; and (4) dilution of the cells to a relatively low density and plating on a solid medium. Adapted from Zimmerman (1993).

BOX 3-2 TELOMERES: A BIOLOGICAL CLOCK REGULATING SENESCENCE AND CELL DEATH

TELOMERES ARE SPECIALIZED STRUCTURES at the ends of linear eukaryotic chromosomes, which are essential for the maintenance of chromosome integrity and protection from exonuclease degradation. They also prevent fusion with other chromosome ends (end-to-end ligation) and thus confer stability to chromosomes. Telomeres are composed of long stretches of tandem repeats of short sequence elements, typically 5–8 bp long, which are rich in G residues on the strand oriented in the 5' to 3' direction toward the end of the chromosome. The G-rich strand (G strand) extends beyond the complementary C-rich strand and terminates as a single-stranded 3' overhang (Fig. 3-11). In a chromosome, the double-stranded telomeric part is looped around and the single-stranded terminus is tucked back inside the double-stranded DNA molecule, forming a displacement loop (D loop). As a result, the telomere terminus is masked and protected from cellular activities that can act on DNA ends. Specific proteins bind to the D loop, possibly allowing its formation or stabilizing it.

The length of telomeres is species specific. For instance, in mice and humans, the telomeres may be 40–150 kbp long, whereas in *Arabidopsis*, they are relatively short, only 2–4 kbp. In animal systems, telomeres are reported to shorten at the rate of ~ 5–10 kbp per cell generation. Once the length has fallen below a critical level, senescence and cell death ensue. Thus, telomere length is thought to act as a biological clock that signals senescence and cell death. In stem cells, in dividing cells in culture, or in cancer cells, telomere length is maintained by several mechanisms, the most important being the activity of an enzyme, telomerase. Telomerase is a reverse transcriptase; it has an RNA subunit that recognizes the base sequence in the telomere and a catalytic unit that adds new G-rich sequences. Telomerase activity is high in proliferating cells, but the activity declines and is undetectable in differentiated, mature cells.

Although most of the above information has been derived from animal cells, plant telomeres are structurally similar to those in other eukaryotes and have a consensus repeat sequence of -TTTAGGG-. Barley telomeres may have several thousand such repeats. In plants there are conflicting reports about shortening of telomeres with differentiation and aging. In barley, they are reported to shorten, paralleled by a decrease in the number of telomere repeats, whereas in some other plants, e.g., *Arabidopsis*, telomere shortening does not seem to occur. However, telomerase activity is high in proliferating cells in meristems, and in cells in culture, and is lost in differentiating cells and is not detected in mature or senescing plant tissues.

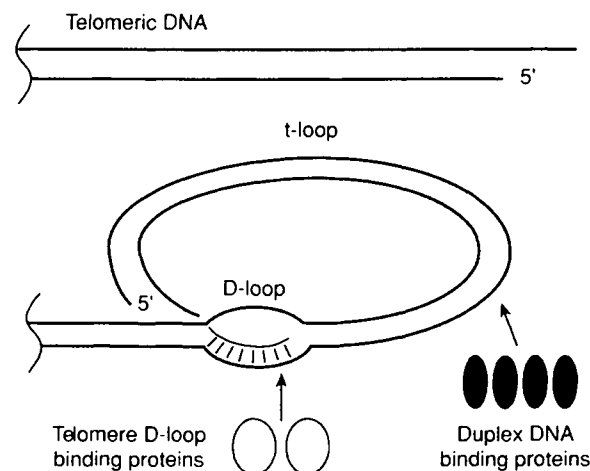


FIGURE 3-11 Structure of a telomere. The telomeric DNA with the 3' overhang of the G strand is shown at the top. The double-stranded part of telomere DNA loops back on itself, forming a lariat structure. The 3' G strand extension invades the duplex telomeric repeats and forms a D loop (displacement loop). Duplex telomere-binding proteins bind along the length of the telomere repeats, and a specialized telomere-binding protein binds the D loop at the junction of the lariat. Modified from Greider (1999) with permission from Elsevier Science.

When explants are taken from plant tissues, wound reaction occurs and a series of events are set in train that are part of the dedifferentiation process (see Chapter 4). Some of the obvious changes that have been recorded include hypertrophy (unusual enlargement) of cells, thinning of walls, loss of stored food, loss of cell- and tissue-specific proteins, and, very important, a change in ploidy levels. Ploidy levels are changed by a variety of methods, including amitotic divisions (nuclear fragmentations), loss of chromosomes at prophase, and segregation of homologous chromosomes (a process that resembles meiosis). These changes in nuclear DNA content are accompanied by cytokinesis, not by the usual process of cell wall formation involving the mitotic spindle and phragmoplast (see Chapter 2), but by the ingrowth of walls from existing ones, a process known as "free wall formation." In later stages of callus proliferation, mitoses followed by normal cytokinesis may occur.

As a result of these processes, the diploid number may be restored in some cells (or lead to new numbers or even the haploid complement, which may be followed by endoreduplication to give the diploid number). At the same time, telomerase activity is restored and progresses at a high rate in proliferating cells. Other changes, less obvious, include a "washing out" or dilution of the cell- and tissue-specific proteins that had been accumulated earlier during differentiation (see Chapter 4). This washing out can occur by cell divisions, both amitotic and mitotic, in rapid succession. Eventually, in this mass, some cells acquire the appropriate genomic and cytoplasmic complement to become potentially embryogenic. Accordingly, totipotency is not just a vague attribute of any plant cell, but rather a specific competence that is acquired by somatic cells or cell lines in culture to reprogram their nucleus and cytoplasm to generate zygote-like cells.

Can these embryogenic cells be recognized? Using a semiautomatic cell tracking system and carrot hypocotyl cells in suspension culture, Sacco De Vries and associates at the Agricultural University, Wageningen, Netherlands, have identified cell types that have a greater potential to form somatic embryos than other cells. According to these authors, cells that are highly embryogenic are spherical and vacuolate or spherical and cytoplasmically rich, but both are small ($\sim 12 \mu\text{m}$ in diameter). Cells that are cylindrical and vacuolate rarely form embryos. The embryogenic cells have also been reported to express a gene that encodes a cell surface-located receptor-like protein, but the target or the function of the protein is unknown. Other studies have correlated embryogenic cells with the production of an arabinogalactan protein (for AGP, see Chapter 2),

which can be localized in the cell wall by staining with its antibody.

8.3. Younger, Less Differentiated Tissues Give Rise to Somatic Embryos More Readily Than Mature or Older Parts

The competence to form somatic embryos is acquired more easily by younger, less differentiated tissues than older, more mature parts, or closer the explant source is to the zygotic embryo. Within the embryo, cells of the shoot meristem have a higher propensity to form somatic embryos than, say, cotyledonary cells. When zygotic embryos are used as the source for explant, there may be little or no callus formation and no exogenous auxin may be required. For somatic embryogenesis in carrot, younger hypocotyls give embryos faster than older hypocotyls. In orchard grass (*Dactylis glomerata*), embryos may be formed from leaf mesophyll directly, without previous callusing if sections are taken near the intercalary meristem at the leaf base; they also require no exogenous auxin (Fig. 3-12). Conversely, if sections are taken from more mature parts, farther away from the intercalary meristem, callusing is induced and a synthetic auxin is required to induce the formation of PEMs. Other fully differentiated and mature tissues from other plants, e.g., phloem parenchyma, leaf mesophyll or epidermis, endosperm or cotyledonary tissue from seeds, pollen grains, may require not only auxin, but also another hormone, a cytokinin, to induce cell divisions.

8.4. The Developmental Program of Somatic Embryos

The developmental program of somatic embryos is very similar to that of zygotic embryos, although there are some differences. Somatic embryos may lack a suspensor and the cell divisions may not be as orderly or patterned as in zygotic embryos, but they go through many of the same stages, preglobular, globular, heart shaped, torpedo shaped, and so on, that zygotic embryos do. Normally, somatic embryos go on to become seedlings and adult plants but, under appropriate conditions, can be made to cease growth and mature, as do zygotic embryos in the seed (see Section 8.5).

What is most interesting is that somatic embryos show expression of many of the same cell- or tissue-specific genes that zygotic embryos do. Thus, the gene for a lipid transfer protein (*LTP1*), which is expressed in protodermal cells of the globular embryos and, later, in epidermal cells of the aerial parts of the adult

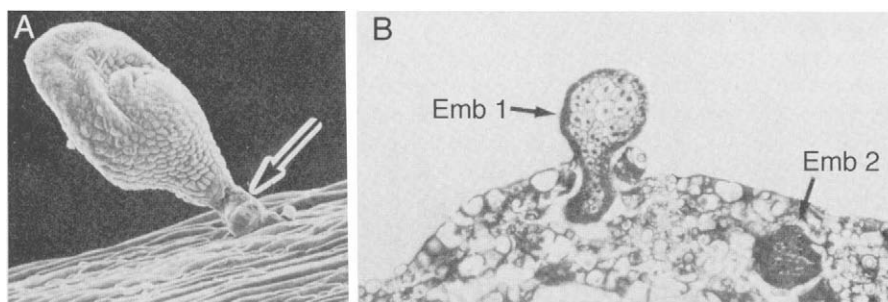


FIGURE 3-12 Production of somatic embryos from orchard grass (*Dactylis glomerata* L.). (A) Scanning electron micrograph of an embryo arising directly from a leaf segment. The embryo is supported above the undisturbed leaf surface by a suspensor (arrow) ($\times 40$). (B) Cross section of leaf ($\times 80$) showing a very young embryo (Emb1) protruding from the leaf surface, with the suspensor originating from the mesophyll, and an embryo (Emb2) beginning to form in the mesophyll. Reprinted with permission from Conger *et al.* (1983), © American Association for the Advancement of Science.

plant, is also expressed in the protoderm of young somatic embryos. AGAMOUS-like 15, a protein belonging to the MADS domain family of transcription factors, accumulates not only in tissues derived from fertilization, i.e., zygotic embryo, suspensor, and endosperm, but also in apomictic embryos and somatic embryos from different sources. Somatic embryos, supplied with exogenous abscisic acid (ABA), synthesize many of the same proteins, both storage proteins and proteins associated with desiccation tolerance, that zygotic embryos do under normal seed development (see Chapter 18). It appears as if the process of dedifferentiation resets the developmental program back to the zygotic state. Somatic embryogenesis, therefore, offers unique experimental material to study the process of dedifferentiation, i.e., the shutting down of the metabolic processes that were current, and a progressive opening of developmental pathways that had been shut down during differentiation.

Because somatic embryos can be produced in large numbers in a more or less synchronous state, they offer a unique material to study biochemical changes, as well as cell- and tissue-specific gene expression during embryo development. Mass production of somatic embryos at near-synchronous stages may also permit the study of cytoskeletal rearrangements and environmental factors associated with patterning.

8.5. Commercial Production of Somatic Embryos

Somatic embryos are clones of the parent from which the explant is taken. Because one can obtain large numbers of embryos from a single explant, they offer a unique method to propagate plants with the desired characteristics of the parent plant, without the genetic mixing that occurs in sexual reproduction (see below). Because somatic embryos in culture continue

to grow and become seedlings, for commercial production it is important to arrest their growth after a certain stage of development. This is accomplished by exposing them to the hormone abscisic acid ($60\text{--}100\text{ }\mu\text{M}$). Embryos given ABA treatment cease growth and mature and, in many cases, also accumulate reserve foods, utilizing nutrients in the culture medium (see Chapter 18). Afterward, they can be embedded in agar and stored until planted. These arrested embryos are sometimes referred to in nurseries as “emblings” (Fig. 3-13).

Although somatic embryos are clones of the parent from which the explant is taken, plants derived from them often show considerable variation in their phenotype from the original parent. This phenomenon is commonly seen in plants derived from old callus cultures and is referred to as “somaclonal variation.” The reasons for somaclonal variation are not fully understood, but they may be related to the cytological and nuclear changes that precede PEM formation (see Section 8.2).

9. CHAPTER SUMMARY

Zygotic embryo development in angiosperms proceeds in diverse ways in different plants in bewildering complexity. To understand it, a beginning has been made by using model systems such as *Arabidopsis*, where the divisions that set up the embryo are highly regular and cell lineages can be followed easily. Genetic studies on *Arabidopsis* mutants reveal that embryo development proceeds through discrete but continuous steps, leading to a blocking of segments, subsegments, and sub-subsegments. Two broad sets of patterns, an apical-basal pattern, which defines the blocking out of major organs, and a radial (or inside-out) pattern, which blocks out the major tissues, are

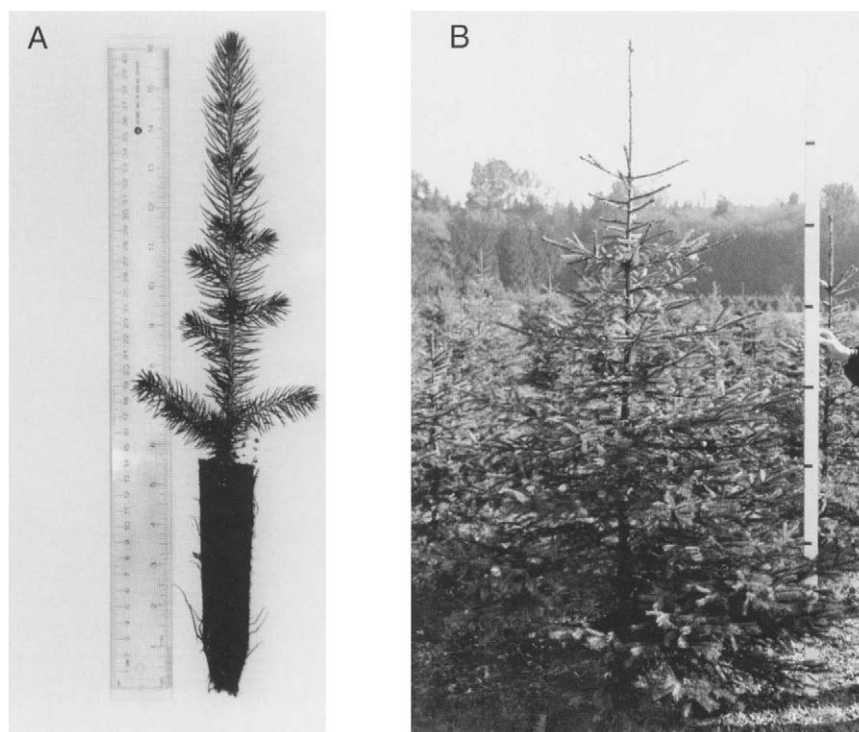


FIGURE 3-13 An embling and nursery of Interior spruce (*Picea englemannii* × *P. glauca*) in British Columbia. Courtesy of BC Research, Vancouver, British Columbia.

involved in embryo development. Several genes related to pattern formations have been cloned. The modes of action of these genes are still not quite clear, but some encode proteins that are transcription factors, which probably regulate broad areas of morphogenesis at the tissue and organ level. Some others seem to be involved in the control of cell division. Still others seem to act as cell surface receptors (see Chapter 25). Although embryo development in angiosperms *in planta* may still be subject to maternal influences (for instance, some maternal genes keep the embryonic program suppressed until after fertilization), evidence from *in vitro* fertilization, apomixis, and somatic embryogenesis indicates that young embryos can set up their own polarity and patterning. Somatic embryogenesis, a unique feature of plants, is a testimony to the phenomenon of open differentiation. It is also a valuable tool for a biochemical dissection of embryogenesis, for the propagation of plants, and for genetic engineering.

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4

Determination, Differentiation, and Dedifferentiation in Plants

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1. COMMITMENT, DETERMINATION, AND DIFFERENTIATION

We have seen in Chapters 1 and 3 that during embryogenesis and subsequent plant development, plant tissues and organs become determined and serve specific functions. In plants it is difficult to decide when “determination” occurs because (i) plants have open growth, they retain apical and lateral meristems, which continue to produce new cells, tissues, and organs throughout plant life and (ii) they show open differentiation, i.e., differentiated somatic or reproductive cells retain the possibility under certain circumstances to revert to an earlier or even the zygotic state. In contrast, in animals, the fate of a cell/tissue is “fixed” at some point in development and the cell/tissue may be considered to be determined at that point.

This chapter discusses the molecular and cellular bases for determination and differentiation, roles of cell lineage and positional cues in determination, what we mean by dedifferentiation, and some of the differences between plant and animal development.

Before doing so, it is important to define the terms determination, commitment, and differentiation. “Determination” is used in this text synonymously with “commitment.” Commitment is defined as being cast in a state where future developmental potentialities become restricted. In molecular terms, this means re-

striction on parts of genome that can be transcribed in a cell or its daughters. Determination is relative. No cell type, including the fertilized egg or zygote, is completely "undetermined"; some cells/tissues are more determined than others, which means that they have a fewer number of future choices open to them than the less determined ones.

Some authors use the terms determination and "differentiation" more or less synonymously. Others have used determination for the blocking out of domains, or organogenesis, as in embryo development, and differentiation for cell- and tissue-specific gene expression and protein synthesis. In this book, the term differentiation is used in the latter sense to denote cell- and tissue-specific gene expression and protein synthesis, but it must be emphasized that the distinction between the two terms is subtle and tenuous. Differentiation is the process whereby cells from a common origin become different, but, as data from pattern formation in embryogenesis show (see Chapter 3), the demarcation of embryonic organs and tissue layers involves a blocking out of domains, followed by subdomains, and sub-subdomains in a progressive manner, leading to a commitment of individual cells for specific fates. A clear demarcation, therefore, as to where determination ends and differentiation begins is not possible, but the context in which they are used usually makes the meaning clear. Differentiation could be considered as the *detectable manifestation* of determination, but "detection" at what level: macroscopic, cellular, or molecular?

2. COMMITMENT OCCURS IN STEPS, AND CHOICES AT EACH STEP ARE LIMITED TO A FEW OPTIONS

We have seen that some polarities and patterns are established very early in development, others are established later, and still others are established even later. Thus, plant development is hierarchical and involves a series of progressive commitments. The progressive nature of commitments means that the derivatives at each step have a more restricted developmental potential than the mother cell; moreover, the number of choices at each step is limited to a few options, usually two.

2.1. Commitment during Embryogenesis

In most embryos, this is illustrated by the first division of the zygote setting out the apical and basal cells, which give rise to the embryo proper and suspensor, respectively (see Chapter 3). Subsequently, a root pole

and a shoot pole are defined in the embryo, and at the shoot pole a shoot apical meristem and cotyledons. At the tissue level, the protoderm is separated from the central cells, followed by the separation of the central cells into ground meristem and procambium, and still later, in roots, of the ground meristem into cortex and endodermis and of procambium into pericycle and vascular tissues. At each step, the choices are limited to two options, and the developmental potentialities of the derivatives are more restricted than those of the parent cell. (Some authors regard commitments as a series of "partitioning" events.) Thus, protoderm, ground meristem, and procambial cells are more determined than the eight-celled proembryo that gave rise to them, and the nature of their derivatives is already determined. Protoderm cells normally will form epidermis, epidermal hairs, guard cells, and elaborate cuticle, but not form xylem or phloem cells. In contrast, procambial cells will normally form vascular tissues, pericycle, and vascular cambium and will not suddenly form glandular epidermal hairs or elaborate cutin. Vascular cambium, when established, is committed to giving rise to derivatives by specific planes of cell division, and the derivatives in turn are committed to forming xylem or phloem cells. In each case, protoderm, procambium, or vascular cambium, the future developmental potentialities are restricted. Plant development, like that of other multicellular organisms, is epigenetic and can be compared to a computer desktop opening of folders within folders, within folders, within folders. . . .

2.2. Shoot vs Leaf Determination

An example of progressive commitment is provided by the development of leaves on shoot meristem. Young leaves can be excised from the shoot apex and placed in a culture medium, and they develop and form a complete leaf. The question arises when does a leaf primordium become committed to being a leaf? Leaf primordia in most flowering plants are difficult to excise at an early enough stage for these experiments to be performed successfully. Analysis of single gene mutants that are defective in some aspect of leaf development indicates, however, that determination occurs at the time the leaf primordium is initiated. Subsequent events in leaf development affect the leaf only, not the shoot meristem, or *vice versa* (see Chapter 3, also Section 4.2.1). In many ferns, however, leaf development occurs over a long period, and leaf primordia of different ages can be excised and put in culture to see whether they will produce only leaves, or shoots. In one such experiment, primordia P1 through P10 were excised and cultured. Results clearly showed that younger primordia were undetermined

TABLE 4-1 Fates of Excised Primordia of *Osmunda* Cultured on Agar Medium Containing Mineral Salts and 2% Sucrose^a

Primordium	Leaves	Shoots ^b	Doubtful or no growth
P1	2	7	11
P2	2	12	6
P3	4	10	6
P4	4	11	5
P5	8	11	1
P6	12	8	0
P7	16	4	0
P8	17	1	1
P9	19	0	1
P10	20	0	0

^aResults from one of several experiments.

^bIn many cases, whole plants were produced (from Steeves and Sussex, 1989).

and produced shoots, both stem and leaves, whereas older primordia were progressively more committed (or determined) to produce leaves only (Table 4-1). These results indicate that, in some ferns, up to a certain time after its initiation, a primordium has the potential to become a shoot, but after that time it becomes committed to develop as a leaf.

2.3. Cell Lineage vs Positional Cues

Many students of plant morphogenesis favor the view that the fate of a cell is determined by the position it occupies in the plant body rather than by its lineage. The critical evidence comes from analyses of genetic mosaics in shoot apices and developing leaves of maize, tobacco, and *Arabidopsis* (for methodology, see Chapter 1). These analyses have revealed that cell lineages, although important, are not a reliable indicator of the eventual fate of a cell. The fate of a cell in plants is determined more by the position it occupies in the plant organ/body than by the manner in which it is derived. This conclusion implies that the commitment to a specific fate is flexible within limits; it is delayed until the position of the cell/organ in relation to its neighbors and the environment is secure. This strategy makes intuitive sense for organisms where cells are fixed in space and cannot move about and where an error in lineage would be difficult to eradicate.

Positional cues imply that environmental factors, as well as existing tissues and organs, exert an influence on the determination/differentiation of new tissues and organs. There are many examples of effects of environmental factors on plant growth and develop-

ment. They are scattered throughout this book. In the following, some examples of interaction with existing cells and tissues are given first before discussing the nature of intercellular communication.

2.3.1. Evidence for Positional Cues

Evidence comes from mutants and from surgical or laser-induced ablation experiments in which a cell layer or tissue is damaged and neighboring cells assume the function of the damaged cells/tissues. Several types of *twin* (*tw*) mutants in *Arabidopsis* are known that exhibit a defect in early embryogenesis. In some mutants, the suspensor cells form a second embryo and polyembryony results (Fig. 4-1). In others, after one or two divisions of the zygote, the apical cell or its descendants arrest, and the basal cell proliferates abnormally, giving rise to a twin or multiple embryos. It is thought that the apical cell and its descendants normally suppress the embryogenic potential of the basal cell and its descendants during early embryo development, a suppression that is released in *tw* mutants. There are other examples of polyembryony in angiosperms; e.g., apomictic (without mixing) embryos result from cells of the embryo sac or nucellus. These cases may also involve a relaxation in the control exercised by the zygote or the young embryo over the embryogenic potential of neighboring cells.

Leaf primordia arise on the shoot apex in strict and species-specific patterns (phyllotaxy). Surgical experiments established a long time ago that if the youngest leaf primordium is damaged, the new leaf primordium arises not in its predicted position, but closer to the damaged one (see Wardlaw, 1968, p. 237).

Cell lineage in roots, in contrast to that in shoots, is highly predictable. Anatomical and clonal analyses of embryonic root in *Arabidopsis* based on cell line-specific markers have been used to develop a "fate map" that defines the location of quiescent center and origin of various tissue layers, such as cortex, endodermis, and pericycle (Fig. 4-2).

Because the origin of different tissue layers is known, surgical or laser beam ablation experiments can provide meaningful data on effects of positional cues on determination. If cells in the quiescent center in a root apex are damaged by a laser beam, the neighboring cells originating from vascular initials assume the characteristics of and become the new quiescent center cells. If cortical initial cells are ablated, neighboring pericycle cells divide periclinally. Of the two daughter cells, one stays in the pericycle and the other invades in the space vacated by the dead cortical initial. Subsequently, the cortical initial divides again to form an endodermal cell and a cortical cell (Fig. 4-3).

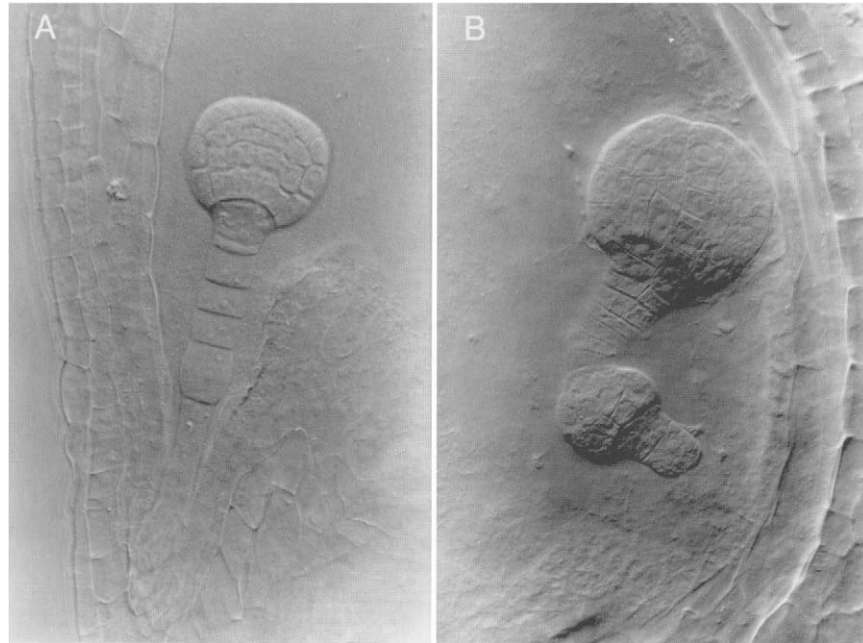


FIGURE 4-1 Wild-type (A) and *twin1* mutant (B) embryos in *Arabidopsis*. Both are at the globular stage. Note the suspensor consisting of a single file of cells in the wild type. In the *twin1* mutant, a second embryo is forming within the suspensor. Scale, width of suspensor is $\sim 20\ \mu\text{m}$. Courtesy of Daniel M. Vernon, Whitman College.

This position-dependent “switch” in the fate of cells supersedes the radial tissue boundaries set during development and implies that positional cues can override the lineage-based determinants in plants. Moreover, the position-dependent signals seem to come from the more mature tissues of the root. For instance, if a particular cell in a cell file, say cortical file, is damaged by a laser beam, the more proximal cells (i.e., farther away from the root apex) still differentiate as cortex. It should be remembered, however, that these changes occur only during development while fate determination is still fluid. Mature cells do not change, except through dedifferentiation (see Section 8).

One of the less known, but elegant example of positional cues and intercellular communication is provided by cambial initials. As explained in Chapter 1, these initials occur as a single layer of cells that divide periclinally to produce secondary xylem (wood) and secondary phloem on two different sides. Although critical studies have not been done, judging from the regularity of annual rings in the wood, it would appear that fusiform initials divide more or less in concert with each other. Assuming a tangential diameter of $40\ \mu\text{m}$ per fusiform initial and allowing a 25% contribution to circumference by ray initials, in a conifer tree with wood cylinder 1 m in diameter, that would work out to about 19,643 fusiform initials in a cross section. Imagine that many cells dividing peri-

clinally more or less in unison! Second, the cambial ring is a site of remarkable adjustments in spatial relationships between fusiform initials and between fusiform initials and ray initials. Initials are constantly being lost by displacement from the initiating ring (they mature as a xylem or a phloem cell) and being replaced by new fusiform or ray initials, which arise by anticlinal divisions. These mutual adjustments allow cambium, and thereby wood or secondary phloem, to adjust to changing conditions in environment. The mechanisms that regulate the divisions of these cells in concert or changes in interrelationships among fusiform and ray initials are unknown, but they must require positional information as well as intercellular communication.

2.3.2. Cell–Cell Communication

How cells communicate with each other is still not understood, although some evidence shows that both plasmodesmata and the cell wall may serve to transmit signals. The nature of signaling molecules is also unclear, although proteins and their mRNAs, cell wall components, and hormones are likely candidates. For instance, the mRNA for *KNOTTED1* gene in maize (see Section 4.2.1) is expressed in shoot meristem in all cells except in the tunica 1 layer (and in founder cells for lateral organs), but the mRNA and the

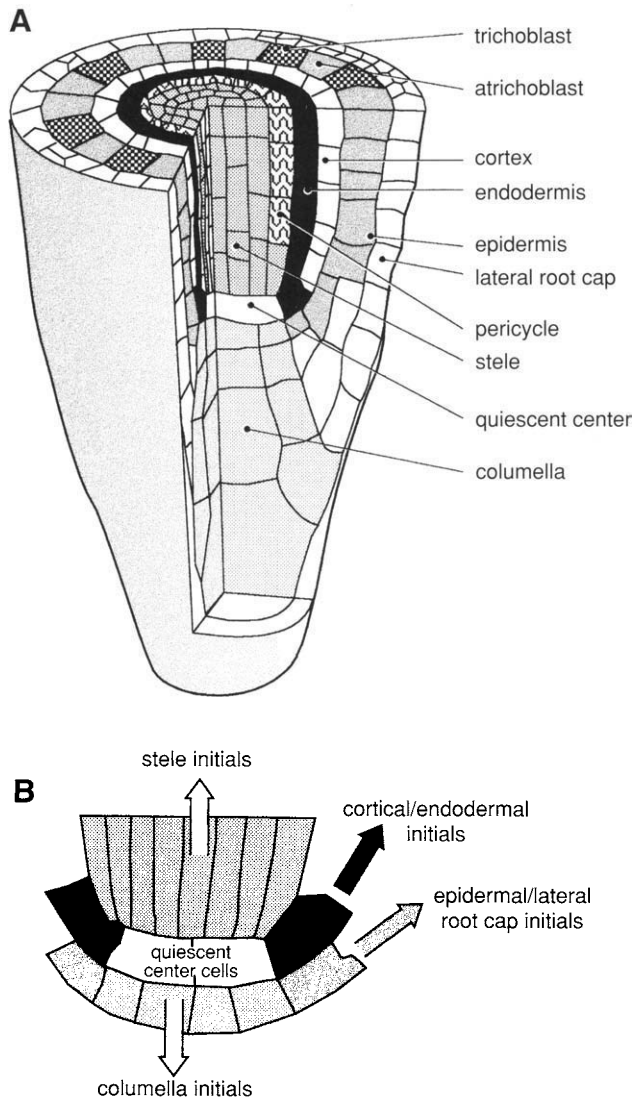


FIGURE 4-2 Schematic drawings showing the three-dimensional structure of the *Arabidopsis* root near its tip (A) and a detail of the quiescent center and surrounding initials (B). A vascular cylinder (stele) is surrounded by single layers of pericycle, endodermis, cortex and epidermis. At the basal end of the root, lateral root cap layers surround the epidermis. The stem-like initial cells in the root meristem surround the quiescent center (see B); they divide to produce two daughter cells, one of which remains as an initial, while the other differentiates further. Separate sets of initials generate columella cells, epidermis and lateral root cap, cortex and endodermis, and pericycle and vascular cells. Distal to the root hair zone, epidermal cells differentiate into root hair-bearing cells (trichoblasts) and nonhair-bearing cells (atrachoblasts). Trichoblasts arise opposite the anticlinal walls separating two subtending cortical cells. In older roots, the organization may be slightly different. From van der Berg *et al.* (1998).

expressed protein are transmitted to tunica 1 layer *via* plasmodesmata (Fig. 4-4). It was mentioned earlier that signals embedded in the cell wall in *Fucus* zygote and two-celled embryo serve to provide cell fate infor-

mation (see Chapter 3, Box 1). Similarly, arabinogalactan proteins in the cell wall of embryogenic cells during somatic embryogenesis and in pollen-stigma/style interaction in flowers are thought to act as recognition molecules for cell-cell interaction. An arabinogalactan protein has also been proposed as a cell-to-cell communication molecule in tracheary element differentiation in *Zinnia* mesophyll cells in culture (for this system, see Section 8.1.3). Hormones have long been considered as signaling molecules for intercellular communication, but an unequivocal demonstration of their role in fate determination is lacking.

3. ROLE OF CELL DIVISIONS IN DETERMINATION

Cell division is inextricably linked with determination and differentiation, but it must be viewed as separate from both. Cell divisions provide a means to compartmentalize and stabilize cytological differences that underlie cell determination or cell differentiation. Supporting evidence comes from mutants that affect cell proliferation without affecting pattern formation or organ fate. For example, *fass* mutants of *Arabidopsis* show an increase in the number of cells in each tissue layer in the globular embryo, but the layers still differentiate according to their prescribed fate. The *aintegumenta* mutation in *Arabidopsis* affects the number of cell divisions in primordia of lateral organs (e.g., leaves, petals), hence their eventual size, without affecting the fate or developmental pattern of the lateral organ. In the *tangled-1* mutation of maize, during leaf development, cell divisions parallel to the long axis of the leaf occur in a highly irregular manner, but still result in organized tissues and cell layers.

Some authors consider all divisions that lead to the production of daughter cells with different fates as asymmetric divisions, whether or not asymmetry is morphologically evident at the time of division. According to this view, any cell division that generates daughter cells with different fates is asymmetric by definition. In this text, however, for convenience of description, a distinction is made between asymmetric and symmetric divisions.

3.1. Asymmetric Cell Divisions

Asymmetric cell divisions have long been considered as a prelude to determination. In Chapter 2, we noted several examples of asymmetric divisions, where daughter cells of unequal size are produced and which inherit different complements of cytoplasm

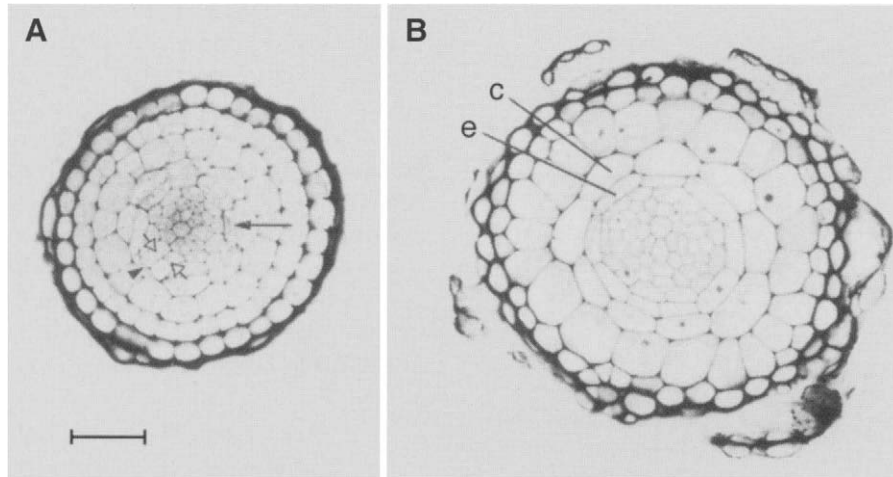


FIGURE 4-3 Ablation experiments in *Arabidopsis* root. (A) Ablation of one cortical initial cell. The dead cell is pressed peripherally (arrowhead) and two pericycle cells are occupying its former position. They have divided periclinally to give rise to cells in both the cortical and pericycle layer (open arrows); the former will divide again to give rise to cortical and endodermal cells. Note the asymmetric division in untreated cortical initials (arrow). (B) Three days after ablation of a cortical initial. The invading pericycle cells have generated a larger cortical cell, (c) and a smaller endodermal cell (e). Two pericycle cells occupy the position of one dead cortical cell. From van den Berg *et al.* (1995).

and organelles (e.g., the first transverse division in the zygote, division separating a trichoblast from an atrichoblast in root epidermis of grasses, formation of stomatal complex in leaf epidermis, formation of companion cells and sieve elements in phloem tissue, formation of generative and body cell in microspores of flowering plants) (Fig. 4-5A, see also Fig. 2-32B). In many of these cases, the cytoplasm and organelles are asymmetrically distributed in the mother cell before cytokinesis. Products of gene activity (mRNAs and/or proteins) may also be asymmetrically distributed in the two segments prior to partitioning, although this

still has to be demonstrated in higher plants (for *Acetabularia*, a green alga, see Section 4 below).

3.2. Symmetric Cell Divisions

Most cell divisions, however, are not asymmetric. Two nearly equal daughter cells that are morphologically indistinguishable are produced, and they become different after cytokinesis. These differences arise by differential gene expression and synthesis of different proteins during G₁ and/or G₂ of the cell cycle. An

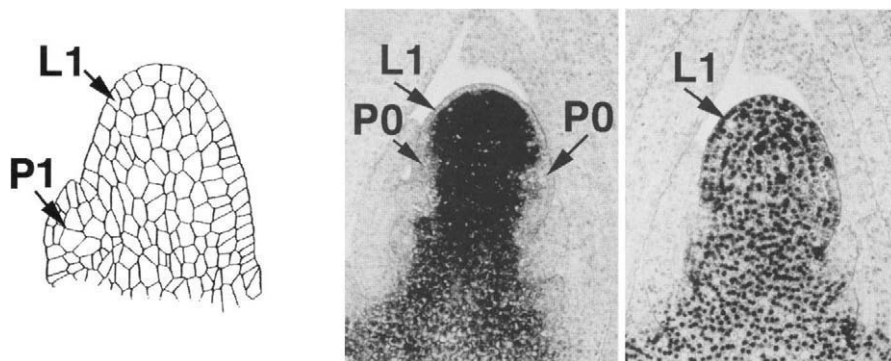


FIGURE 4-4 Movement of KNOTTED1 protein in shoot meristem of maize. (Left) Schematic drawing of the shoot apical meristem with a distinct outer cell layer, L1, and a newly initiated leaf primordium (P1). (Center) Visualization of KNOTTED1 mRNA expression by *in situ* hybridization. Expression occurs throughout the meristem, except for the L1 layer and in the founder cells for the leaf primordium (P0). (Right) Immunolocalization of KNOTTED1 protein. The protein is localized in nuclei, but, unlike the mRNA, is seen in the L1 layer. It is thought that the KNOTTED1 mRNA and protein both are translocated *via* plasmodesmata. From Jackson and Hake (1997).

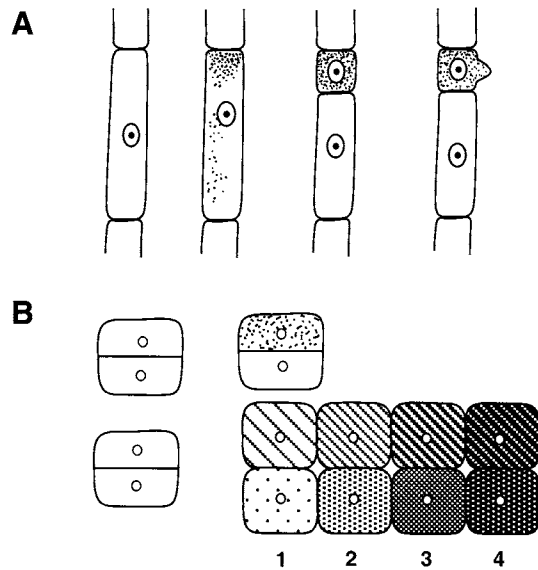


FIGURE 4-5 Models showing cytological differentiation associated with asymmetric and symmetric divisions. (A) An asymmetric cytokinesis is preceded by partitioning of the cytoplasm into dissimilar halves. (B) Two scenarios after symmetric, formative divisions. In the model on top, two cells become different after one division. In the model on the bottom, progenies of two cells become progressively different.

accumulation of discernible morphological differences in these cases may occur abruptly after a single cell division or may occur gradually over several cell cycles and lead to a progressive channelization of a cell's potentialities (Fig. 4-5B).

How two daughter cells immediately after a symmetric division come to express different genes or how the cytoplasm becomes asymmetrically organized before an asymmetric division are mysteries about which we still know very little. Data on *in vitro* fertilization in maize and on axis formation in zygotes of *Fucales*, reviewed in Chapter 3, clearly suggest that cytoplasmic asymmetries can be, and indeed are, established in previously symmetrically disposed cells. In *Fucus*, environmental factors cause changes in cytoskeleton, which, in turn, affect the cytoplasm and organelles to become asymmetrically disposed in the cell. The cytoskeleton probably plays a similar role in creating cytoplasmic asymmetries in higher plant cells, but this assumption needs to be confirmed. Cell-to-cell communication may also be involved.

4. MECHANISM OF DIFFERENTIATION

In a multicellular organism, cells and tissues serve specific functions, functions for which they have been

specialized. The functions are performed efficiently and to the benefit of the whole organism, but at the price that the specialized cells, tissues, and organs have only limited parts of their genome open for transcription. Thus, a typical leaf mesophyll cell, which is specialized for photosynthesis, may have 40–50 well-differentiated chloroplasts, about 10^6 molecules of photoreceptors, chlorophylls a and b, about the same number of associated CHLa and CHLb proteins, and 10^8 molecules of ribulose 1,5-bisphosphate carboxylase/oxygenase (RUBISCO), the principal carbon-fixing enzyme. In contrast, a root parenchyma cell, which is not doing much else besides storing starch, will have no chloroplasts, no chlorophyll and associated proteins, and no RUBISCO; instead it would have amyloplasts (starch-storing plastids) and large amounts of ADP glucose pyrophosphorylase, one of the major starch-synthesizing enzymes in nonphotosynthetic tissues. The root and mesophyll cells have the same genomic DNA, but they are specialized for different functions because different genes are expressed in the two types of cells.

Differential gene expression is used here in a broad sense to include all gene-directed activity, not only gene transcription, but also posttranscriptional and posttranslational modifications, as well as gene silencing. Differential gene activity is the basis for the phenomenon known as epigenesis, the unfolding of the developmental program of an organism (Waddington, 1953).

Gene activity involves at least three types of genes: (i) housekeeping genes that encode proteins required for general housekeeping, such as enzymes involved in respiration, sugar uptake, or synthesis of proteins or synthesis/replication of nucleotides and polynucleotides; (ii) genes that are expressed in a cell- and tissue-specific manner and which encode proteins that are specific for the channelized route or the designated function; and (iii) regulatory genes that specify pattern or that regulate the expression of cell/tissue specificity. At the same time, there is a progressive “shutting down” of parts of genome that are not going to be necessary for the destined function of the cell. Shutting down, in this context, means not being available for transcription.

Housekeeping genes need no further discussion.

4.1. Cell/Tissue-Specific Genes

There are numerous examples of cell- and tissue-specific gene expression. In the example of mesophyll cells given earlier, genes encoding the small and large subunits of RUBISCO, as well as chlorophyll a and b proteins, are expressed in photosynthetic tissues, not

in storage parenchyma of roots. Seeds accumulate a number of different kinds of storage proteins. Storage protein genes are expressed selectively in seed tissues, not in vegetative tissues of the plant. The *PHENYLALANINEAMMONIA LYASE* (*PAL*) gene encodes the enzyme phenylalanine ammonia lyase, which catalyzes an important step in the synthesis of phenolic compounds, including lignin. It is expressed in cells that differentiate as lignified xylem elements, but not normally in parenchyma cells of the pith or cortex. Hydroxyproline-rich glycoproteins (HRGPs) and glycine-rich glycoproteins (GRPs) are deposited in cell walls. Genes encoding the protein moieties of HRGPs are expressed in most cell types, but those encoding the protein moieties of GRPs are expressed selectively in differentiating xylem elements, not in other cells. The *LIPID TRANSFER PROTEIN* (*LTP*) gene encodes a protein that mediates the transfer of lipids to the cell exterior, most likely for the deposition of cuticle. The *LTP* gene is expressed in the protoderm layer of embryo and epidermal cells of aerial parts of the seedlings of *Arabidopsis*, but not in other cells of the embryo, roots, or subepidermal cells in the aerial parts of the seedling.

4.2. Regulatory Genes

Most regulatory genes in plants encode transcription factors or proteins involved in signaling, such as receptor-like kinases. As explained in Appendix 1, there are many different kinds of transcription factors encoded by multigene families. They include homeobox genes that encode homeodomain-containing proteins; genes that encode proteins with MYB domains, or AP2 domains, or MADS-box domains. Many transcription factors specify patterns of future development by regulating the expression of structural genes that encode cell-/tissue-specific proteins, while some modify the action of other transcription factors. The role of genes encoding receptor-like proteins in plants is still conjectural. These proteins are probably involved in the perception of environmental and/or hormonal signals, including signals from neighboring cells. In the following, some examples of regulatory genes involved in the determination of identity, or size, of shoot meristem are provided.

4.2.1. Shoot Meristem Identity

Plant homeobox genes are required for cell fate determination, as well as pattern definition and specification of organ/tissue boundaries. They are classified into several families based on analysis of the homeodomains and other conserved motifs. One family,

the *KNOX* (for knotted-like homeobox) family of genes, named after the maize *KNOTTED1* (*KN1*) gene, is further divided into two classes, I and II. Class I *KNOX* genes are expressed in shoot meristems and are downregulated in primordia of lateral organs. For instance, the *KN1* gene in maize and its ortholog *STM* gene in *Arabidopsis* (an ortholog is a homologous gene in a divergent taxon) are first expressed in the globular-heart stage embryo in cells of the presumptive shoot apex, but not in cotyledons (scutellum in maize). Subsequently, they are expressed in both vegetative and floral shoot meristems of the adult plant, but, remarkably, they are not expressed in cells on the periphery, which are the founder cells of lateral organs, such as leaves or petals (see Fig. 4-4). Other members of the class I *KNOX* family in rice, tobacco, *Arabidopsis*, and spruce show similar expression in the shoot apex, not in lateral organs.

Analysis of mutants, as well as the constitutive expression of these genes in transgenic plants, indicates that class I *KNOX* genes are involved in the demarcation and maintenance of the shoot meristems. For example, a loss-of-function, recessive mutation (*stm* in *Arabidopsis*) results in an embryo with no or rudimentary shoot meristem (Fig. 4-6A). A dominant mutation *kn1* of maize is characterized by the ectopic formation of "knots" over lateral veins in leaf blades. *Hooded*, a dominant mutation of barley *HvKNOX3*, results in the formation of florets on the awn of the lemma, the first lateral organ of the floret. Overexpression of *STM* or *KN* homologues in transgenic plants results in the ectopic placement of meristems in leaves (Figs. 4-6B-D).

Class II *KNOX* genes have a more varied pattern of expression and their roles are unknown.

4.2.2. Floral Meristem and Organ Identity

Floral meristems express other regulatory genes that distinguish them from vegetative shoot meristems. They also express regulatory genes that determine the identity of floral organs, i.e., whether a sepal, petal, stamen, or carpel is formed. In dicots, many of these genes, although not all, belong to the MADS-box gene family. To give an illustration, *LEAFY* (*LFY*), a non-MADS-box gene, and *APETALA1* (*AP1*), a MADS-box gene, in *Arabidopsis* encode transcription factors that act as primary determinants of floral meristem identity. *AP1* also specifies an organ, the sepals whorl in *Arabidopsis* flower. Loss-of-function mutations in these genes (e.g., *lfy*, *ap1*) lead to a partial conversion of presumptive floral sites into shoots (Fig. 4-7). Transgenic gain-of-function plants in which *LFY* or *AP1* is constitutively expressed show a partial to complete

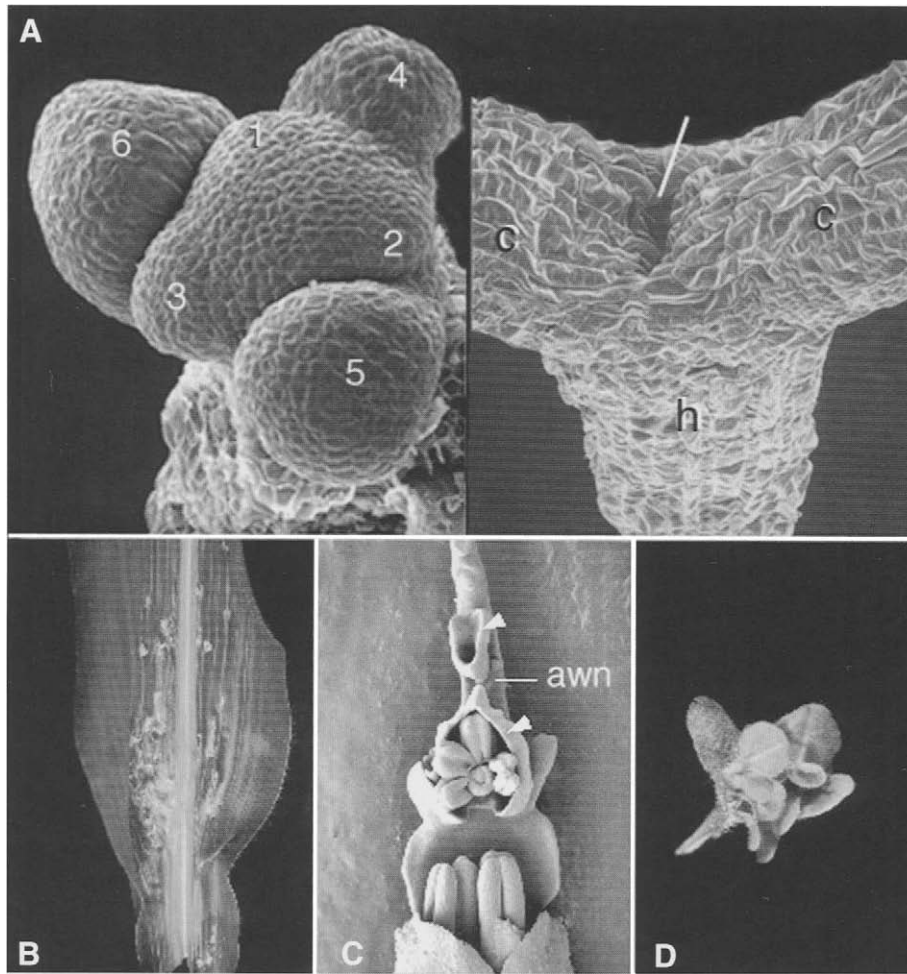


FIGURE 4-6 Phenotypes of plants mutated in or misexpressing class I *KNOX* genes. (A) Wild-type (left) and *stm5* mutant (right) of *Arabidopsis*. The wild type shows a floral shoot meristem in a plant ~ 4 weeks old. Primordia for six flowers, numbered 1 (youngest) through 6 (oldest), are shown. The *stm5* is a strong allele, and plants usually do not develop beyond the seedling stage. Parts of two cotyledons (c) fused at the base and hypocotyl (h) are shown. The presumptive site of the shoot apex is indicated by a line. Weaker alleles show a small or rudimentary shoot meristem and survive to adult plants. With permission from Endrizzi *et al.* (1996), © Blackwell Science Ltd. and courtesy of Thomas Laux. (B) Leaf blade of *kn1* mutant of maize showing knots forming on the lamina. (C) Flower from a Hooded barley plant showing ectopic floral meristems (arrowheads) forming on the awn. (D) Tobacco leaf from a transgenic plant overexpressing *KNAT1* with ectopic meristems. From Reiser *et al.* (2000) with kind permission from Kluwer.

transformation of all shoots into flowers, suggesting that these genes are largely sufficient to promote flower meristem identity. Mutational analysis indicates that *AP1* acts downstream of *LFY*, in agreement with its role in determination of the sepal whorl. The expression of *LFY* transcripts precedes that of *AP1* in wild-type plants. Orthologs of *LEAFY* and *APETALA1* are known from snapdragon (*Antirrhinum majus*).

4.2.3. Size of Shoot Meristem and Lateral Organs

In meristems, cell divisions in stem cells are balanced against the number of daughter cells that enter

the differentiation pathway. In shoot apical meristems, this balance determines the size of the meristem. If the number of proliferative divisions in the central zone (CZ) exceeds the number of cells that enter the peripheral zone and the formation of lateral organs, proliferative cells accumulate in the CZ, leading to a larger size of vegetative shoot (including floral) meristems than in wild-type plants. In the reverse case, the shoot meristem undergoes a diminution (cell enlargement plays a limited role in shoot meristem size determination). The *WUCHSEL* (*WUS*) gene in *Arabidopsis* is necessary for the maintenance of stem cells in the CZ throughout development, and *wus* mutations lead to

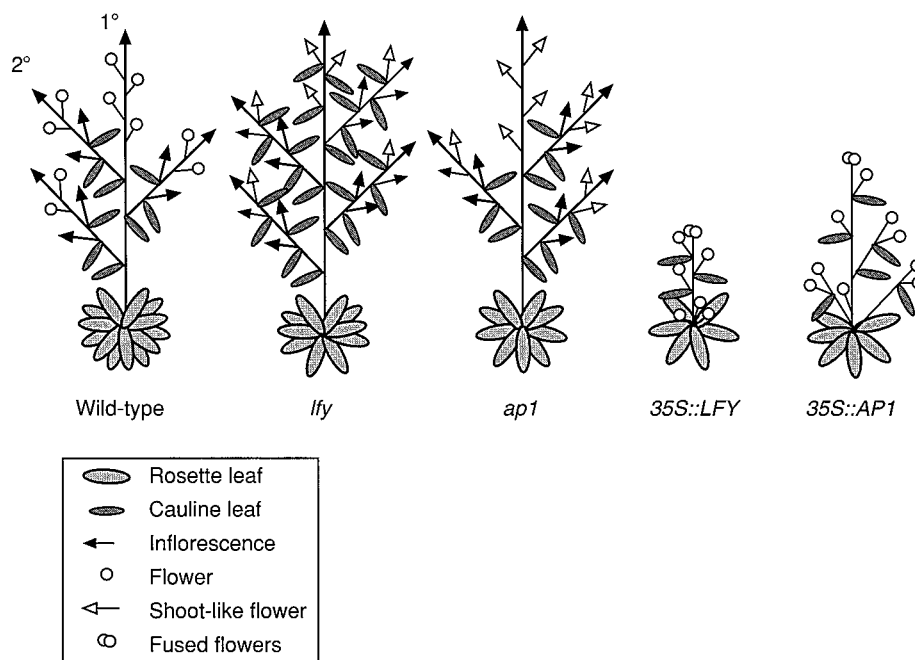


FIGURE 4-7 Genes controlling floral meristem identity in *Arabidopsis*. Mutations in *LEAFY* (*LFY*) and *APETALA1* (*AP1*) lead to a partial conversion of flowers into vegetative shoots, whereas transgenic plants, which constitutively express *LFY* or *AP1* under the control of a constitutive promoter (*35S::LFY*, *35S::AP1*), show a partial to complete transformation of shoots into flowers. Positions of a primary (1°) and a secondary (2°) shoot meristem are indicated for the wild-type plant. From Liljegren and Yanofsky (1996).

the termination of meristem activity. In contrast, the *CLAVATA* (*CLV*) genes in *Arabidopsis*, seem to promote organ formation at the periphery. In *clv1* and *clv3* mutants, this activity is disturbed, leading to a gradual increase in the size of shoot meristems. In the *clv1* mutant, the defect becomes evident in the embryo and continues through vegetative and inflorescence development; eventually the shoot meristems of *clv1* may be over a 1000-fold larger by volume than those of the wild type (Fig. 4-8). Thus, *WUS* and *CLV1* act antagonistically to maintain the shoot meristem size. Genetic analysis suggests that *WUS* is a putative target for *CLV1* regulation (see also Chapter 25). As mentioned earlier, the *SHOOT MERISTEMLESS* (*STM*) gene is required for shoot meristem identity. Both *STM* and *WUS*, like *CLV1*, are expressed throughout plant development and act competitively to maintain shoot meristem activity.

The *CLAVATA1* gene is expressed in cells of the central zone in shoot meristems, but not in primordia of lateral organs. It encodes a receptor-like protein, which could perceive positional information regulating cell division rates or transition to differentiation (for more details on signaling by *CLAVATA1* protein see Chapter 25). In contrast, *AINTEGUMENTA* (*AIN*) is expressed in lateral organs, such as cotyledons,

leaves, and petals, but not in shoot meristems. It regulates the size of lateral organs by regulating the duration of cell division. In *ain* mutants, lateral organs are significantly smaller than in the wild type because cell divisions cease prematurely. The *AIN* gene encodes a transcription factor of the AP2 domain type.

Many other regulatory genes are known that specify broad patterns of organ development or tissue/cell identity in leaves, roots, or flowers. Some of these genes are considered in other chapters. It is important to note, however, that mutations in regulatory genes can affect wide sectors of plant development, and if the genes are expressed constitutively, they alter the normal patterns of development by altering the expression patterns of their target genes. One of the major gaps in our knowledge still pertains to how the regulatory genes, such as *KN1*, *STM*, *LFY*, *AP1*, *CLV1*, and *AIN*, act to regulate shoot meristem identity or the morphology of shoot meristem or lateral organs.

4.3. Shutting Down of Parts of Genome

There are numerous examples of parts of genome not being transcribed during determination/differentiation. For instance, genes for seed storage proteins

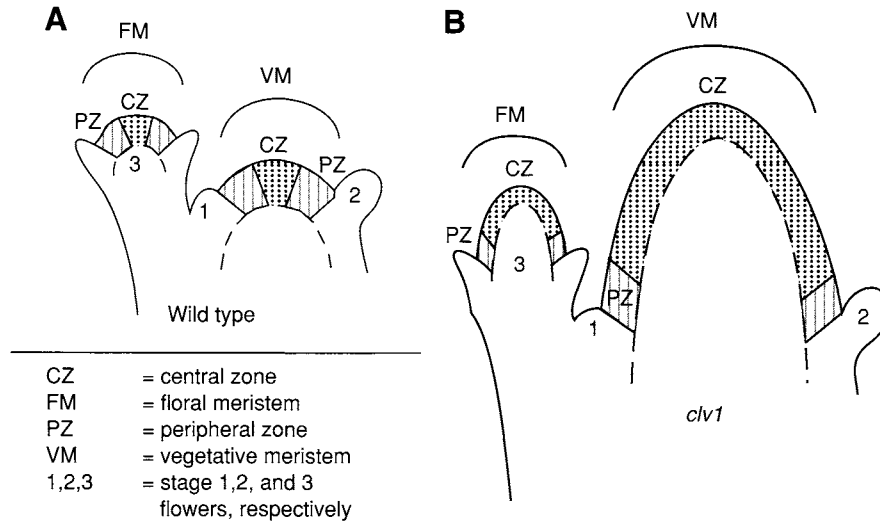


FIGURE 4-8 Diagram of wild-type (A) and *clv1* (B) vegetative and floral shoot meristems. Idealized longitudinal sections through inflorescences of the wild-type and *clv1* mutant of *Arabidopsis*. CZ, stem cells in the central zone; PZ, organ-forming peripheral zone. From Clark *et al.* (1997).

are shut off and not transcribed in vegetative tissues; many genes associated with chloroplast development and photosynthesis are normally not transcribed in nongreen tissues. An elegant example of selective shutting down of gene function comes from tissue culture work using explants from different tissues of the same plant—stem cortex and leaf mesophyll. In some cultivars of tobacco (e.g., “Havana” 425), callus cultures derived from the stem cortex have no requirement for exogenous cytokinins (CKs), a class of plant hormones, they are CK autonomous, whereas cultures derived from leaf mesophyll require exogenous CKs, they are CK dependent. Somatic embryos, produced from the two sources by appropriate cultural and hormonal treatments, go on to produce mature plants, but explants from stem cortex of the new plants are still CK autonomous, whereas those from leaf mesophyll show CK dependence (see Fig. 4-12). It appears as if the leaf mesophyll cells lose the potential to synthesize cytokinins during their differentiation, whereas the stem cortex cells retain that capability. Pith cells from the stem show an intermediate condition (see Section 6.3 below). Clearly the genes for the production of cytokinins are present in the plants, but they seem to be silenced during the development of leaf mesophyll, whereas they remain active in cortical tissue and some pith cells. Thus, in the same plant, different tissues are stabilized in different states with regard to CK biosynthesis during their differentiation.

In summary, differential gene expression and suppression combine to make determination and differentiation of body parts, tissues, and individual cells

possible. The unfolding of this basic developmental plan is in the first instance determined by the genomic make up of the organism, but, as discussed in Section 5 below, there is reason to believe that cytoplasm also plays a role in determination.

5. ROLE OF CYTOPLASM

Several studies indicate that the cytoplasm of a cell, at least in part, determines which genes will be expressed. This evidence comes from transplant experiments in which the nucleus from a mature differentiated cell is introduced into an enucleated oocyte or zygote or a grafting experiment where the nucleus of one cell type is exposed to the cytoplasm of another cell type. For instance, in animal systems, terminally differentiated muscle or kidney cells in culture produce only their own differentiated cell type, i.e., they cannot produce progeny destined to have other functions, such as nerve or intestinal cells. However, using amphibian oocytes, it can be shown that a nucleus from a muscle cell transplanted into an enucleated egg cytoplasm will generate all major cell types of a complete embryo. More recently, nuclei from fully differentiated cells implanted in enucleated eggs have been used to produce embryos in sheep and other mammals. These experiments indicate that the nucleus of a differentiated cell is conditioned in its expression by its own cytoplasm, which is released when it is placed in an oocyte.

Acetabularia acetabulum is a single-celled green alga that in its vegetative state is morphologically differentiated into a basal rhizoid and an axially elongated stalk, which bears whorls of branching hairs. The single diploid nucleus resides in the rhizoid. At reproductive onset, an elegant cup-like cap develops at the apex, giving the alga the nickname Mermaid's Wineglass (Fig. 4-9A). Different *Acetabularia* show species-specific morphologies of the cap. These cells are large enough (species vary from 1 to 10 cm) and heal readily enough to permit grafting experiments.

Grafting and amputation experiments with *A. acetabulum* have revealed several interesting features about the interaction between the nucleus and the cytoplasm. First, the morphology of the cap is determined by the nucleus. If the nucleus from one species is transferred to an enucleated individual of another species, the cap that develops has the morphological characteristics of the rhizoid donor. Second, differentiation of the cap is mediated by putative long-lasting transcripts, which migrate up into the stalk, forming a gradient in the process. The cytoplasm in the stalk carries the information required for morphogenesis. If the adult stalk is divided into enucleate segments, the apical segment usually forms a cap at its apical end; the midsegments grow but do not undergo mor-

phogenesis. The rhizoidal segment regenerates a new stalk (Fig. 4-9B) and eventually a cap.

A mutant, *kurkku* (Finnish for cucumber), does not undergo the morphological transition from juvenile to adult phase; apparently, it lacks some gene product necessary to complete vegetative and reproductive phases. If a *kurkku* enucleate stalk is grafted to a wild-type rhizoid or, conversely, the rhizoid to a wild-type stalk, caps form in both cases. The wild-type cytoplasm is sufficient to provide whatever is needed for reproduction.

These results indicate that the cytoplasm can carry enough information to specify patterns of morphogenesis. Electron microscopy, combined with transplant experiments, reveals that a nucleus transferred from a mature plant to the enucleated rhizoid of a young plant acquires the physical characteristics of a young nucleus. In contrast, a nucleus from a young plant transferred to the rhizoid of an old mature plant looks like a mature nucleus. These results, similar to those from nuclear transplant experiments in frog oocytes, indicate that the cytoplasm can affect the morphological characteristics, and presumably the function, of nucleus.

Transplant experiments with egg cells of angiosperms and gymnosperms have not been performed because of their general inaccessibility, but, as mentioned in Chapter 3, it is now possible to isolate

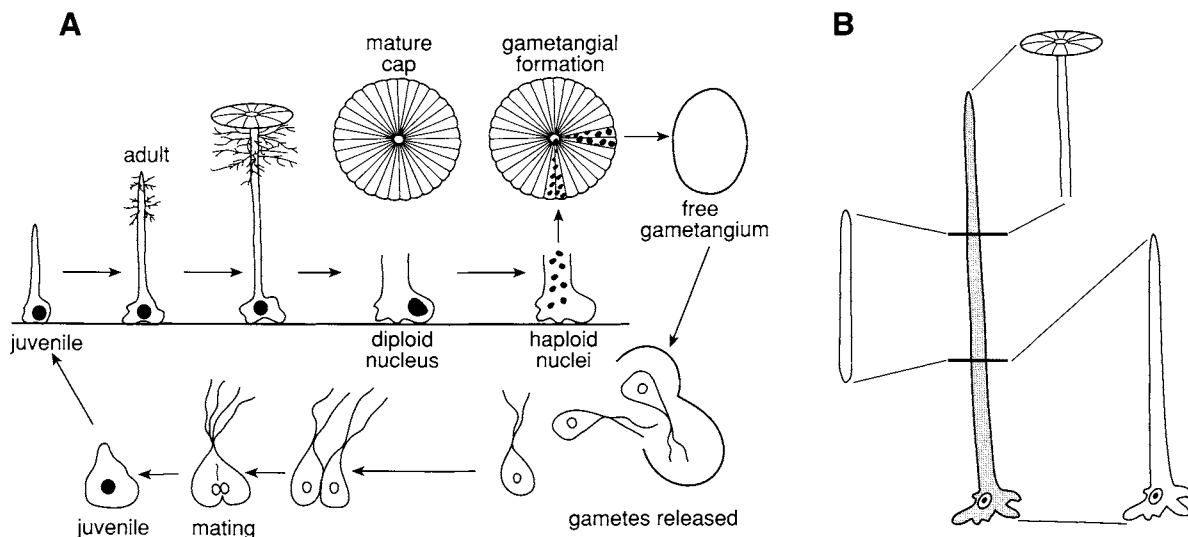


FIGURE 4-9 Life cycle of *Acetabularia* and results from some grafting experiments. (A) A zygote develops into a large regionally differentiated cell with a rhizoid, a long stalk, branching hairs arranged in whorls, and a cap with incomplete partitions arranged like the spokes of a bicycle wheel. The single diploid nucleus resides in the rhizoid until the cap is nearly fully developed. Then the nucleus undergoes meiosis, followed by several rounds of mitoses. Hundreds of haploid nuclei are formed, which migrate upward into the cap segments where gametangia (spheres in which gametes are made) are formed. Mature gametangia release biflagellate gametes that fuse to form a zygote. Modified from Berger *et al.* (1987). (B) Regeneration experiments with cut segments of stalk. From data in Mandoli (1998).

egg and sperm cells from angiosperms and carry out *in vitro* fertilization. Thus, it is possible to conduct experiments in which the nucleus from a highly differentiated cell, such as a mesophyll cell, is transplanted into an enucleated egg or zygote, and *vice versa*. Results of such experiments will be awaited with great interest.

6. STABILITY AND TRANSMISSION OF DETERMINED STATES

The stability of determined states is seen not only in meristems, but also at different stages/phases of plant development, as well as in plant propagation through cell culture lines.

6.1. Determined State of Meristems

The determined state of shoot and root meristems, and vascular cambium, was discussed in Chapter 1. To recapitulate, in these meristems, also intercalary meristems in grass leaves, the number of stem cells is increased by proliferative divisions. In formative divisions, of the two daughter cells, one retains the characteristics of stem cell, whereas the other is set on the pathway to further differentiation. Thus, the stem cells remain stabilized in their state of determination through repeated cell divisions.

6.2. Phase Change in Plant Development

Shoot development in plants is characterized by what are known as phase changes. Many perennial plants show a juvenile phase and an adult phase when flowering occurs. For instance, in English ivy (*Hedera helix*), the juvenile plant is a creeping vine with palmate-lobed leaves and alternate phyllotaxy (Fig. 4-10). The adult flowering plant is an erect bush with entire ovate leaves and spiral phyllotaxy. The change is usually abrupt, but transitional forms are known. Plants can be propagated from cuttings. If cuttings are taken from a juvenile plant, they give rise to plants with juvenile habit; if taken from adult plants, they give rise to plants with adult habit.

Annuals also show different states of determination along their stems. Lateral (axillary) buds along the primary axis in tobacco plants (*Nicotiana tabacum* cv Wisconsin 38) are stabilized at different physiological states when produced. The difference can be demonstrated by monitoring the growth pattern of these buds if they are released from apical dominance by decapitation of the main axis. Tobacco plants produce terminal

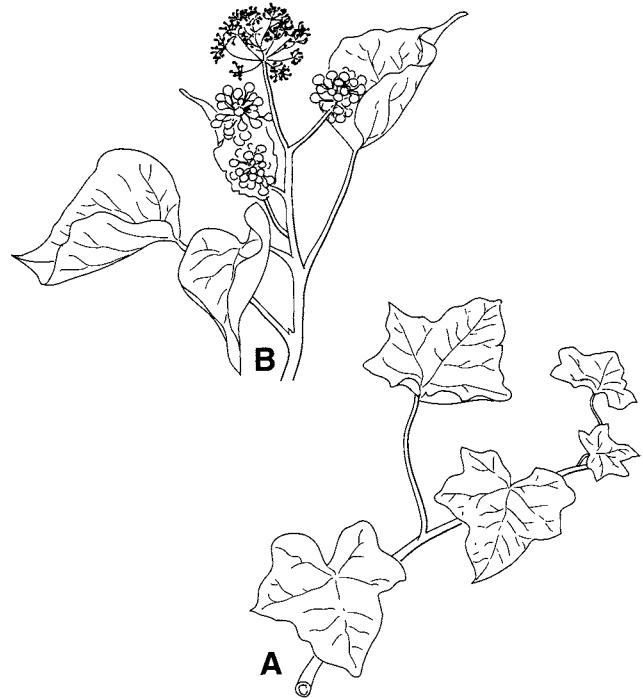


FIGURE 4-10 Juvenile (A) and adult (B) forms of English ivy (*Hedera helix*). From Meins and Binns (1979).

flowers. If decapitation is performed after anthesis of the terminal flower, lateral buds at successively higher positions on the stem produce fewer and fewer vegetative nodes before flowering than the more basal ones (Fig. 4-11). However, if decapitation is affected before the primary axis is florally determined, lateral buds produce the same number of vegetative nodes before flowering as the main axis would have if not decapitated. Thus, the behavior of axillary buds in tobacco is specified both by positional information provided by the primary axis and by the developmental state of the shoot at the time the bud was initiated.

6.3. Habituation

As mentioned earlier in Section 4.3, cells/tissues (e.g., pith and mesophyll of tobacco) become stabilized in their differentiated state and, when put in culture, require cytokinin and/or auxin for cell proliferation and growth. Under certain conditions, however, this stabilization can be disturbed. In culture lines from pith parenchyma of tobacco, "Havana" 425, provision of cytokinin over several cultures occasionally leads to production of a line that is CK autonomous, a phenomenon referred to as "habituation." These habituated lines remain CK autonomous in culture after culture, but may revert to being CK dependent (Fig. 4-12). Leaf mesophyll cells, however, never become

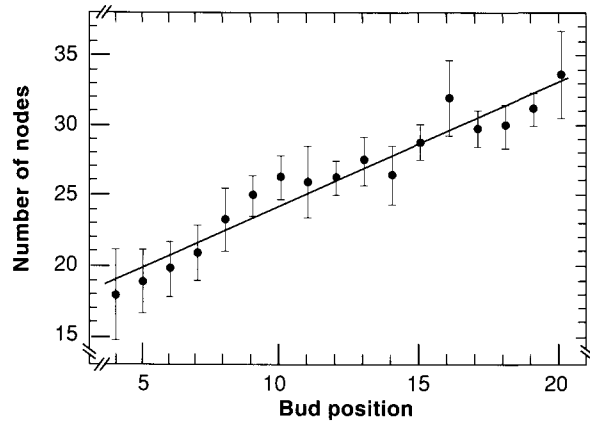


FIGURE 4-11 Position-dependent growth of axillary buds on field-grown *Nicotiana tabacum* cv Wisconsin 38 plants. Bud positions are numbered from the top down, with bud number 1 being the first axillary bud immediately below the inflorescence. At anthesis of the terminal flower, plants were decapitated in the internode above the indicated bud, and the number of vegetative nodes produced before flowering was plotted. As can be seen, the closer an axillary bud is to the terminal flower (or later it was produced in plant development), the fewer number of vegetative nodes it produces before flowering. Each data point is an average of vegetative nodes produced by four buds at each position \pm SD. From McDaniel *et al.* (1989).

habituated. CK autonomy can also be obtained in pith cultures grown only with IAA, without kinetin, if the temperature is raised to 35°C, about 10° above the standard culture temperature. A similar habituation of culture lines to auxins, vitamins, or antioxidants has been described. It seems, therefore, that the mechanisms used to shut down parts of genome during differentiation, while being highly stable, can nonetheless be broken under certain circumstances.

These states of determination passed on from cell to cell or generation to generation are examples of developmental (i.e., epigenetic) changes in which different states are maintained by self-perpetuating alterations in the pattern of gene expression. They do not cross the meiotic barrier. The developmental program is reset at the time of meiosis.

7. HOW IS THE DETERMINED STATE MAINTAINED?

The determined state is maintained by a combination of several mechanisms, among which histones and DNA methylation play a predominant part. Other mechanisms include what is referred to here as “cytoplasmic imprinting” and, in plants, polyploidy.

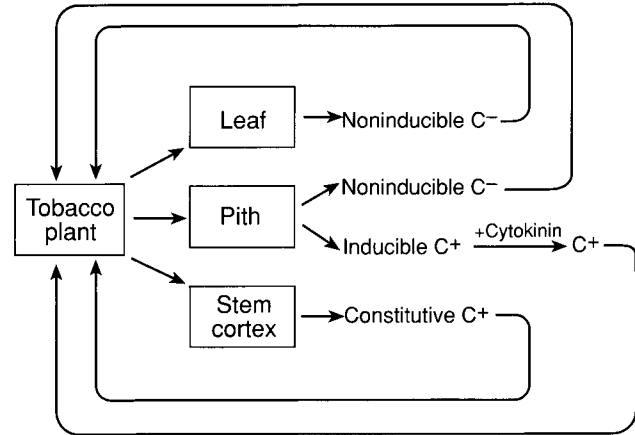


FIGURE 4-12 Schematic representation of cytokinin (CK) requirement of cultured tobacco (*Nicotiana tabacum*) cells from stem tissues. Cortical cells placed in culture do not require exogenous CK for proliferation; they are constitutively C^+ (CK autonomous or CK producing). In contrast, leaf mesophyll cells in culture always require exogenous CK for proliferation; they are C^- or cannot be induced to produce CK. Pith cells are stabilized in two states. One state, like leaf mesophyll cells, is C^- and cannot be induced to produce CK. The other state is inducible and becomes CK autonomous, if supplied with exogenous CK. Each cell line can give rise to the whole plant and persists indefinitely. From Meins (1989).

7.1. Histones

Histones are basic proteins involved in the packaging of eukaryotic genomes into nucleosomes and higher order packaging of chromatin, as well as interactions with nonhistone proteins. A major posttranslational modification of core histones is their acetylation. Acetylation occurs at specific lysine residues at the N-terminal tails of histones and is carried out by histone acetyl transferases (HATs) (Fig. 4-13). Although the reasons are not well understood, acetylation is associated with the derepression of genes, probably because it prevents the condensation of nucleosomes into higher order packaging. Transcriptionally active euchromatin domains tend to be relatively highly acetylated, whereas inactive heterochromatin domains are consistently underacetylated. Also, if the synthesis of a core histone, such as H4, is inhibited (this can be accomplished by putting the *H4* coding sequence under the control of an inducible promoter and providing noninducible conditions), there is a decline in nucleosome density and a correlated increase in the number of genes expressed.

Histone deacetylases are enzymes that remove acetyl groups from the core histones. Thus, they reverse the action of HATs and play a key role in the repression of transcription. HATs and histone deacetylases belong to gene families with many members in each, which show species- and tissue-specific expression.

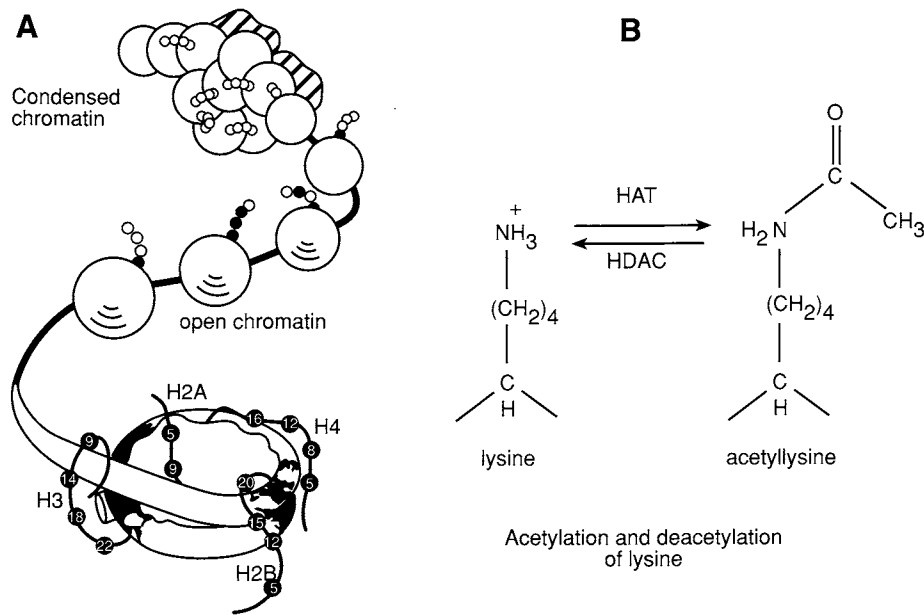


FIGURE 4-13 A schematic illustration of open and condensed chromatin conformations and acetylation/deacetylation of histones. (A) The lower part of the figure shows the nucleosome core particle, consisting of eight histones (two each of H2A, H2B, H3, and H4) surrounded by DNA. The N-terminal tail domains of the histones protrude from the DNA coils (for clarity, only four of the eight tails are shown) and are subject to various posttranslational modifications, including acetylation of selected lysines. Open and condensed chromatin conformations are shown in the middle and upper parts of the figure. It is thought that the N-terminal tail domain of H4 interacts with an acidic patch of an adjacent nucleosome, thereby encouraging chromatin condensation. Acetylated lysines (shown as solid discs, some are numbered) in the H4 tail inhibit such interaction, whereas unmodified lysines (open discs) facilitate it. Chromatin condensation also involves interaction with nonhistone proteins, which may themselves interact with the histone tails. From Turner (1999). (B) Acetylation and deacetylation of histones. Histone acetyltransferases (HAT) transfer an acetate group from acetyl CoA to the ϵ amino groups of specific lysine residues, which favor open chromatin. Histone deacetylases (HDAC) reverse this acetylation. From Johnson and Turner (1999).

7.2. DNA Methylation

Among the known modifications of DNA during development, methylation of the pyrimidine ring of cytosines is the most prevalent. DNA methylation occurs almost universally in eukaryotes (exceptions are fission yeast and fruit fly). It is recognized as the major agency responsible for inactivation of an endogenous gene, a transposon, or a foreign piece of DNA (e.g., transgenes, viral DNA). Inactivation results either at the transcription level or posttranscriptionally. A methylated cytosine(s) in the promoter region can prevent transcription by impeding the recognition of promoter sequences by transcription factors; methylation in the coding sequence may result in defective transcripts, which are degraded. In either case, gene action is prevented.

DNA methylation serves a dual role. By inactivating transposons or foreign DNA, it eliminates interference with the expression of the endogenous program. By silencing endogenous genes, it helps in maintenance

of the determined/differentiated state. Maintenance of the determined state requires that patterns of methylated cytosines be recognized and transmitted without change to daughter cells. This is accomplished by the activity of a family of conserved DNA methyltransferases, which are specific to cell/tissue and species. Methylation occurs in nascent strands after DNA replication by a process known as maintenance methylation, which transfers the particular pattern of unmodified or modified cytosines from a parent to a daughter cell unchanged (Fig. 4-14). Changes in methylation patterns occur by *de novo* methylation or by passive demethylation through the failure of maintenance methylation.

The transmissibility of methylation patterns to daughter cells explains the stability of cells that are determined but are still dividing and produce derivatives that differentiate, e.g., cells in apical or lateral meristems. It explains how fully differentiated cells that have ceased division, as in mesophyll or root storage parenchyma, segregate their gene activities to produce RUBISCO and chlorophyll a and b proteins in

mesophyll cells or ADP glucose pyrophosphorylase in root cortex, respectively.

Methylation can also be lost. Reprogramming of methylation occurs at the time of meiosis, but diploid cells in culture progressively lose methylation; 1 to 5% of methyl groups may be lost per cell division. In contrast, permanent lines either do not lose it or maintain the level by constant remethylation. The loss in methylation coupled to cell divisions may explain the phenomenon of habituation referred to earlier (Section 6.3) and the acquisition of totipotency by differentiated cells during somatic embryogenesis (see Chapter 3).

7.3. Polyploidy

Many differentiated or mature plant cells exhibit endopolyploidy. C values of 4, 8, 16, 32, 64, and even 128 C are common in storage parenchyma cells of cotyledons (e.g., soybean), leaf epidermal cells, and trichomes. While the exact function of polyploidy in maintenance of the determined state is not known, polyploid cells in differentiated tissues are known to require hormones, such as cytokinins, for the induction of cell division. A lack of adequate concentrations of hormones in polyploid cells can maintain them in a determined or differentiated state.

7.4. Cytoplasmic Imprinting

A combination of synthesis of regulatory proteins, cell/tissue-specific proteins, and the shutting down of parts of genome by histones and DNA methylation probably results in qualitative changes in the cytoplasm. The expression "cytoplasmic imprinting" is used for these changes, it is a characteristic feature of cell determination and differentiation. Because the determined state is maintained during proliferative divisions in meristems, it follows that cytoplasmic imprinting continues during such divisions.

8. DEDIFFERENTIATION AND REDIFFERENTIATION

Chapter 1 considered some examples of reversal of established commitments, or dedifferentiation. Dedifferentiation means a programmed change in the metabolic machinery of a cell, shutting down of genes that were being transcribed in connection with the established function of the cell, and adjustment to new conditions. This is usually followed by induction of new sets of genes and their transcription—what may be called redifferentiation. Dedifferentiation and

redifferentiation include a release from the determined state, or a series of determined states, one after the other, and opening of and commitment to new developmental programs. In the simile of computer desktop folders, this means shutting down the document that is currently open and moving back to the folder that contained it, opening the other document, or moving back to an earlier, or an even earlier, folder.

8.1. What Happens during Dedifferentiation?

While the phenomenon of dedifferentiation has been known for a long time to plant biologists, "dedifferentiation" has been a vague term, "whatever we may mean by it," as F. C. Steward said in 1958. Our insight into the phenomenon is even now not much better, partly because most studies have concentrated on the forward process of cell differentiation; very few have concentrated on the reverse process of dedifferentiation. To compound matters, because an experimental approach usually involves excision or cutting of plant tissue, the effects of trauma of cut, or "wound response," are also involved. It is not easy to separate dedifferentiation from wound response, and in the following the two are considered simultaneously.

There are very few studies that have systematically examined what happens during dedifferentiation. Common manifestations of dedifferentiation include loss of stored food in the case of storage organs, such as cotyledons, when excised and placed in culture; thinning of walls and even polar or amoeboid growth; a change in ploidy levels, leading to a diploid, even a haploid, complement brought on by a variety of processes accompanied by free wall formation, as well as cytokinesis (see Chapter 3). Not all these changes may be evident in a specific case. Thus, there are degrees of dedifferentiation where some involve cell divisions and others do not.

8.1.1. Change in the Metabolic State of a Cell

Jerusalem artichoke (*Helianthus tuberosus*) produces large fleshy tubers that serve for the storage of inulin. Discs can be punched out from tuber slices and used to study cell expansion under the influence of the synthetic auxin 2,4-D. In preparation for the assay, tubers are sliced, discs punched, and then left in water for 24 h, a period known as "aging." During aging, several cellular changes become evident; most notably, there is a proliferation of ribosomes, dictyosomes, and endoplasmic reticulum, changes that involve the expression of many genes that had been hitherto silent.

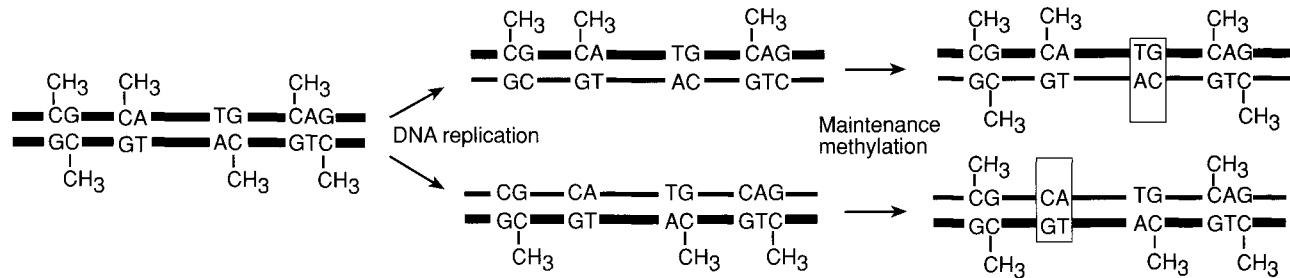


FIGURE 4-14 Transmission of methylation patterns through a cycle of DNA replication. Patterns of methylation based on cytosines located in symmetric sequences (CpG and CpNpG) are transmitted to both daughter strands following replication by the action of a methyltransferase, which preferentially methylates hemimethylated DNA at the replication fork, a process known as maintenance methylation. Methylation of cytosines in nonsymmetric sequences (CpXpX where X is any base other than G) is not transmitted to the newly synthesized daughter strand. Parental strand, thick line; newly synthesized strand, thin line. Loss of methylation at nonsymmetric sites on the daughter molecules is indicated by a box surrounding these residues. From Finnegan *et al.* (1998).

These changes are evident as early as 8 h after excision, even though the cells do not become responsive to exogenous 2,4-D until after 24 h. This indicates that certain metabolic changes must occur in storage parenchyma cells before they can enlarge in response to an exogenous auxin.

8.1.2. Dedifferentiation of Mesophyll Cells

Mesophyll cells are highly differentiated cells where certain proteins associated with photosynthesis, such as the small and large subunits of RUBISCO and CHL_{a,b} proteins, are highly expressed, whereas many other genes, such as those associated with the cytoskeleton or lignin biosynthesis, e.g., tubulin genes and PAL genes, respectively, are expressed little or not at all. In one study, changes in mRNA and protein levels of small and large subunits of RUBISCO were followed in asparagus (*Asparagus officinalis*) mesophyll cells that had been isolated and kept in culture. It was found that the mRNA for the small subunit of RUBISCO (encoded by a nuclear gene) was undetectable within 1 day and that of the large subunit (encoded by plastidic DNA) decreased to low levels within 2 days postisolation. Because of a lack of new synthesis, the corresponding proteins gradually declined in abundance, falling to basal levels by days 6 to 7, which coincided with the onset of rapid cell division. The chloroplast rRNA levels also declined to basal levels by day 6. Chloroplasts showed little change in size or morphology for the first 8–9 days of culture, after which they divided to form small proplastid-like structures. This study indicates that major changes in *Asparagus* mesophyll cells, occur in chloroplast RNA and proteins before cells are committed to division.

8.1.3. Dedifferentiation and Redifferentiation of Tracheary Cells

Tracheary cells are a model system for cell differentiation (see Chapter 2, Section IV. 3). They can be made to differentiate in cell populations from many systems, although the cell expansion stage may be bypassed in some cases. The various systems include regeneration of xylem (and phloem) cells in pith and cortical parenchyma cells after wounding or severance of a vascular strand, differentiation of tracheary cells in callus tissue derived from soybean cotyledons, in cortical discs from pea roots, in lettuce pith explants, in Jerusalem artichoke root discs, and in isolated single mesophyll cells from *Zinnia* leaf (see also Chapter 14). Considerable debate has raged whether cell division is a prerequisite for such differentiation, but now it seems clear that at least in some systems, cell division may not be necessary. For instance, it has been shown by time-lapse photography that isolated *Zinnia* leaf mesophyll cells can differentiate directly into tracheary cells without any prior cell division. Such differentiation proceeds, as *in planta*, via stages that are similar to a procambial cell and immature xylem tissue.

Because *Zinnia* mesophyll cells can be prepared in large number in a more or less synchronous state and because differentiation into tracheary elements (TEs) can be induced in ~50% of cells by suitable hormonal treatment (see Chapter 14), this system has become a favorite material for a study of *in vitro* TE differentiation and associated gene expression. Tracheary differentiation in this system proceeds in three stages (Fig. 4-15). The first stage corresponds to a period of dedifferentiation during which the cells lose their potential to function as photosynthetic cells, the actin network that holds the chloroplasts in place at the

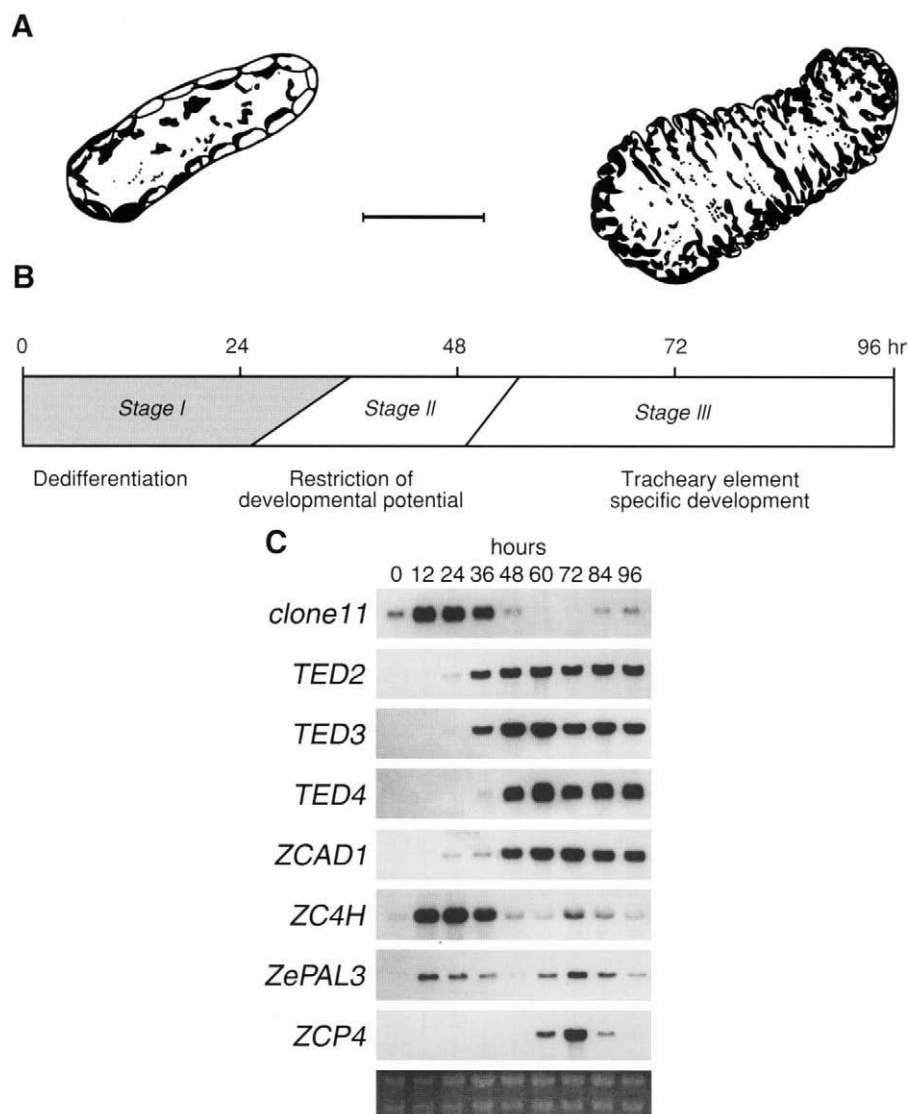


FIGURE 4-15 Dedifferentiation of mesophyll cells and induction of tracheary elements (TEs) in *Zinnia elegans*. (A) A single mesophyll cell just after isolation (left) and a differentiated tracheary element with a sculptured secondary wall (right). Bar: 30 μ m. With permission from Shinohara *et al.* (2000), © National Academy of Sciences, USA. (B) Stages in TE differentiation and their approximate timing. Stage I corresponds to a period of functional dedifferentiation in which isolated mesophyll cells lose their photosynthetic ability. Stage II represents a period during which the developmental potential of the cell is restricted and defined to express genes that are specific to immature xylem tissue. In stage III, changes specific to TE differentiation, which is secondary wall deposition and lignification, occur, followed by programmed cell death. Modified from Fukuda (1997). (C) Expression of selected stage-specific genes during TE differentiation. Stage I: *clone 11* (a cDNA clone whose corresponding transcripts are expressed during stage I). Stage II: *TED2*, *TED3*, *TED4* (TE-differentiation-related genes), and *ZCAD1* (a gene encoding cinnamyl alcohol dehydrogenase). The functions of *TED* genes are unknown, but they are specifically expressed during immature xylem tissue differentiation. Stage III: *ZePAL3* (a gene encoding phenylalanine ammonia lyase), *ZC4H* (a gene encoding cinnamate-4-hydroxylase), and *ZCP4* (a gene encoding cysteine protease). Note that *ZC4H* and *ZePAL3* among stage III genes are expressed in stage I as well. These genes may be involved in wound-induced biosynthesis of phenylpropanoids in stage I and in lignification in stage III. Cysteine protease is involved in final autolysis of the protoplast. Also note that the stage II genes shown here continue to be expressed through 96 h. This is because some populations of TE precursor and immature xylem cells differentiate into TEs, whereas others continue to exist as they are. Total RNA was isolated from *Zinnia* mesophyll cells that had been cultured for indicated times in a TE-inductive medium, and RNA gel blot hybridization was performed using gene-specific probes. Ethidium bromide-stained bands of rRNA are shown at the bottom as an internal control to demonstrate equal loading of RNA. Modified from Yamamoto *et al.* (1997).

periphery of the cell is dissolved, and the chloroplasts lose their photosynthetic ability. During stage II, cells become progressively committed to TE differentiation and begin to express various genes that are expressed in early stages of xylem tissue differentiation. In the third and final stage, cells deposit a secondary wall, which is lignified, followed by autolysis of protoplast and cell death, features typical of TE differentiation.

8.1.4. Dedifferentiation Involving Cell Divisions

Other examples of dedifferentiation include the induction of adventitious roots in stem or leaf cuttings, induction of shoot buds in roots, and somatic embryogenesis by differentiated cells. These systems allow a study of cell dedifferentiation, induction of cell divisions, and establishment of new developmental pathways. However, the information on these systems, especially dedifferentiation, is still very fragmentary. In rooting of apple stem cuttings, cells with swollen nuclei and dense cytoplasm appeared 24 h after cutting in the regions of stem where roots are to be formed. The first cell divisions were observed after 48 h, and meristemoids of ca. 30 cells were formed after 96 h. Thus, several changes in the cytology of cells precede cell division.

Production of somatic embryos from mature tissues involves loss of stored food, if present, thinning of walls, and a change from a polyploid to a diploid complement, all accompanied by rapid cell proliferation. In addition, an activation of telomerase activity and restoration of telomere lengths (see Chapter 3, Section 9) and probably global demethylation of nuclear DNA are involved.

8.2. Cell Divisions Provide a Mechanism for Resetting the Developmental Program

The examples just given indicate that dedifferentiation always results in shutting down the existing program, but subsequent changes progress to varying degrees. In some cases involving a few or no cell divisions, only a close alternate or preceding pathway may be opened, changing the developmental program slightly. Examples of such partial dedifferentiation include cytological and nuclear changes during "aging" in Jerusalem artichoke root discs and tracheary cell differentiation in *Zinnia* mesophyll cells. Rooting in stem or leaf cuttings or formation of shoot buds on roots represents a greater degree of dedifferentiation. Complete dedifferentiation, as occurs under rapid cell proliferation during somatic embryogenesis, results in

a loss of cytological imprinting, restoration of the nuclear DNA content to the diploid state, and restoration of the genomic DNA to that of the zygotic state, such that the embryogenic program can be reinitiated.

8.3. Determination in Plant Cells Is Not as Tightly Controlled as in Animal Cells

Various forms of regeneration of tissues and organs, and vegetative propagation, all involving dedifferentiation and redifferentiation to varying extents, are common in plants because they confer a selective advantage to organisms that are rooted and cannot escape adverse environmental conditions. Determination maintains the cells in a metastably differentiated state, but it is not tightly controlled and allows reversions to earlier or alternate states of commitment relatively easily with changes in the environment or intercellular signals.

By comparison, in animal development, once a cell has differentiated and expressed its cell-type-specific genes, it and its daughters never normally change to another cell type. Liver, muscle, and nerve cells placed in culture continue to produce their kinds. Cells transplanted to a heterotopic site in the embryo and surrounded by cells of a different type still continue to retain their differentiated state, i.e., produce their cell-specific proteins. Animal cells can nevertheless show limited reversion to an earlier state, as when cells from adult tissues are grown in culture and form blastema. As Fig. 4-16 shows, differentiated myocytes will revert to being myoblasts in culture, and divide indefinitely, but then redifferentiate as myocytes only.

Plants also have very few organs and tissue types compared to animals (Fig. 4-17). As a result, reversion to earlier steps or all the way back to zygotic state is relatively easy.

As reviewed in the section on cell cycle in Chapter 2, there also seem to be major differences between differentiated animal and plant cells with respect to the strategy they use to enter the cell cycle. Differentiated animal cells, and "quiescent" yeast cells, are held in G_0 , their entry into the cell cycle requires specific cues, and the cyclins and their cognate CDKs are specifically synthesized. In contrast, plant cells, although differentiated and specialized, are maintained in G_1 or G_2 , and although cyclin synthesis is necessary, the CDC2 mRNA is present in all meristems and continues to be present in moderate amounts in mature parenchyma cells, pericycle cells, and others. It is as if mature and determined plant cells are held in a state of semireadiness for cell division.

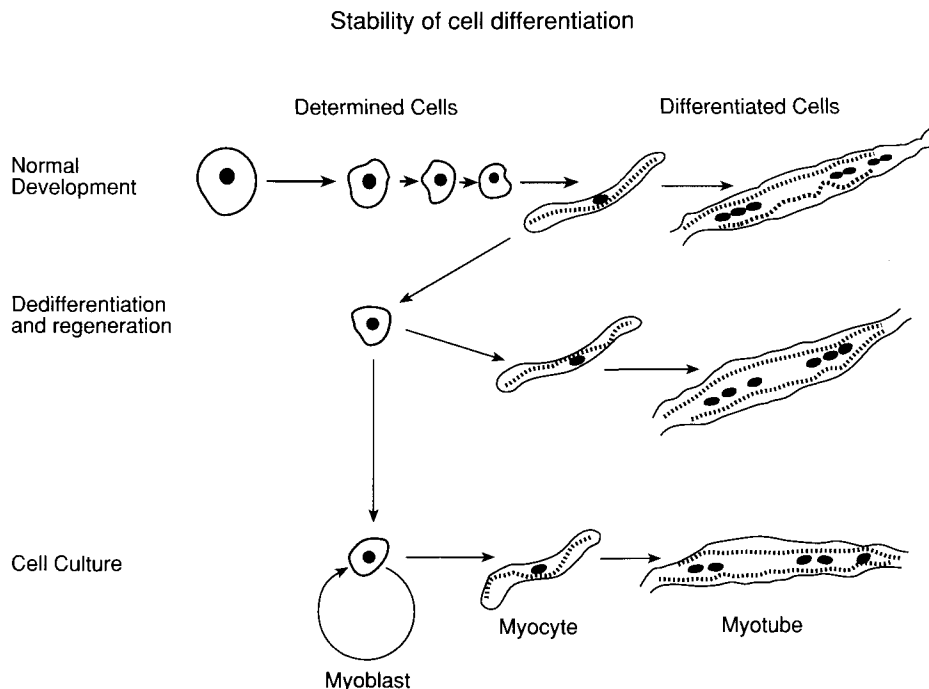


FIGURE 4-16 Muscle cells in culture can show partial reversion to an earlier state. Myoblasts are determined cells that give rise to myocytes, which are single muscle cells, each with a myofibril; several myocytes fuse together to form a myotube. Myocytes (but not myotube) can dedifferentiate and go back to myoblast in culture, divide indefinitely, and again redifferentiate as myocytes and myotubes. From Gurdon (1992).

These differences in body organization, state of commitment, and readiness of mature cells to divide reflect differences in strategies for survival that plants and animals have adopted. It is unknown whether these differences between plant and animal development reflect qualitative or quantitative differences.

9. CHAPTER SUMMARY

Determination or commitment occurs in a stepwise, hierarchical fashion with limited choices at each step and results in blocking out of domains, subdomains, and sub-subdomains in a progressive manner. In plant

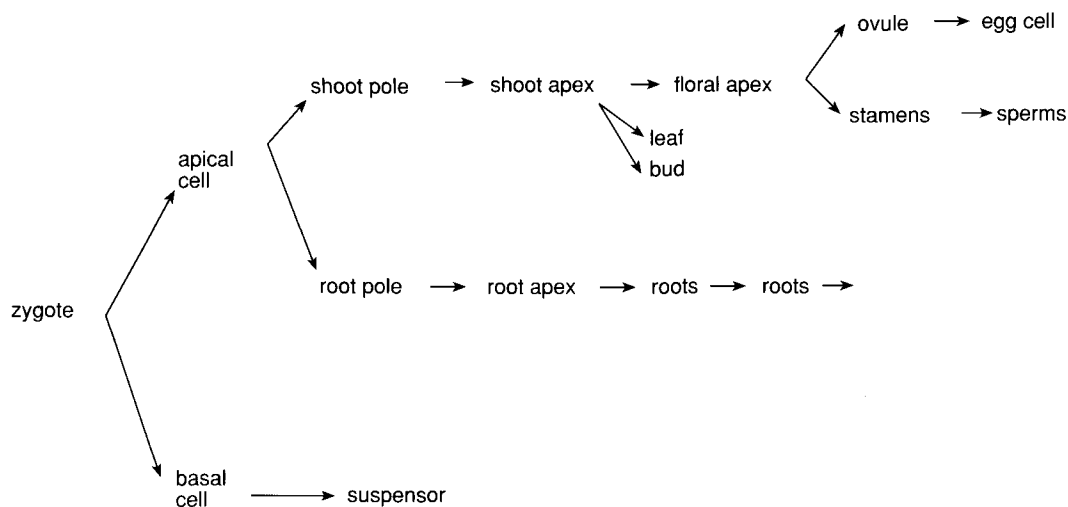


FIGURE 4-17 A summary diagram of organogenesis in plants.

development, cell lineages, although they indicate how a cell is derived, are often a poor indicator of the eventual fate of the cell. The position of the cell in relation to other cells in the plant body seems to play a greater role in fate determination than cell lineage. Such positional information requires cell-to-cell communication, but the nature of such signaling is not fully understood. Differentiation is the process whereby cells of common ancestry become different. Determination and differentiation are made possible by the differential activation of regulatory genes and cell-/tissue-specific genes. Many regulatory genes encode transcription factors or proteins involved in signaling. These proteins define and demarcate broad areas of organ development and/or tissue/cell fate. Tissue- and cell-specific genes encode proteins responsible for specific functions of a cell or tissue. Other factors that play a role in differentiation include cytoplasmic factors, but their nature is not fully understood. The determined state is maintained by a combination of factors, including histone-modulated chromatin rearrangements, DNA methylation, polyploidy, and what is referred to here as "cytoplasmic imprinting." The determined state in plants is not as rigorously controlled as in higher animals. It is kept in a metastable state, i.e., it can change under appropriate conditions. Dedifferentiation always shuts down the existing program, but subsequently proceeds to varying degrees. It may open only a close alternate or preceding pathway; at the other extreme, it may result in a total loss of cytological imprinting, restoration of genomic DNA to the zygotic state, and reinstatement of the embryogenic program. While some processes of dedifferentiation may not involve cell divisions, a re-setting of the embryogenic program always involves rapid cell divisions. Plants have relatively few organs and tissue types compared to animals. Plant cells, although differentiated and specialized, are maintained in G₁ or G₂ in a state of semireadiness for cell division. These features, combined with the metastable state of determination/differentiation, confer adaptive advantages to organisms that are rooted and cannot escape their environment.

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Molecular and Genetic Tools for Study of Plant Development

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1. INTRODUCTION

Molecular and genetic techniques are very powerful tools in the study of plant development and its regulation by environment and hormones. They are outlined in this Appendix because they have general applicability to most topics covered in this book.

2. REGULATION OF GENE EXPRESSION

Plant development, like that of any other organism, involves differential gene expression. By that we mean that while all cells of an organism have the same genomic make up, different genes are expressed in different cells, tissues, and organs. Such differential expression is controlled by the developmental program of the organism as modified by environmental and hormonal cues. To give an example, α -amylases are enzymes involved in hydrolysis of starch. Plants have many different types of α -amylases, which are encoded by multigene families; some are expressed in leaves, some in tubers, some in seeds. In cereals, such as wheat and barley, members of two families are expressed following seed germination under the control of a class of hormones called gibberellins (GAs). mRNAs of members of the two families are induced at different times and at different concentrations of GA (see Chapter 19). In wheat, a third group of α -amylase genes is expressed during seed development, not during seed germination, and not under the control of GA. Other gene families present in other plant parts/tissues are expressed at different times in development, but have no specific relationship to GAs. How are these genes differentially regulated in the same tissue and in different tissues/parts of the same plant? To understand this, let us look at the structure of a hypothetical gene and how its transcription is regulated, but first let us be clear about what we mean by gene regulation.

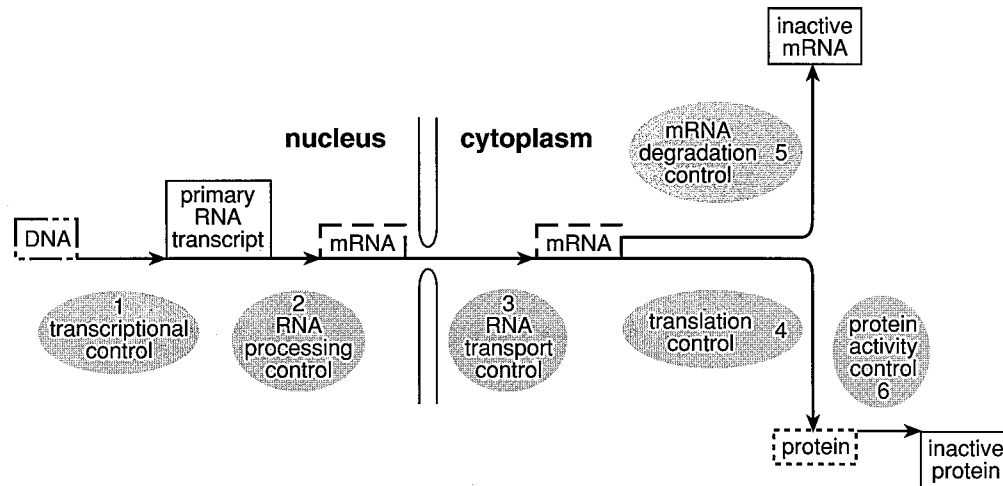


FIGURE A1-1 Regulation of gene expression. Regulation can occur at any one (or more) of the following six steps: (1) Transcription of the gene to yield the nascent transcript. (2) Processing of the transcript to yield mRNA. (3) Transport of the mRNA. (4) Translation of the mRNA to yield the nascent polypeptide. (5) Stability of the processed transcript. (6) Posttranslational modifications and stability of the finished protein. Strictly speaking, regulation of gene expression means control at the transcriptional level, i.e., step 1. Reprinted with permission from Alberts *et al.* (1994), © Garland Publishing, Inc.

Gene expression can be regulated at many different levels (Fig. A1-1). It can be regulated at the level of transcription, at the levels of processing of nascent transcripts, or stability of processed transcripts. It can occur at the level of translation, at the level of post-translational modifications in the polypeptide, or stability of the finished protein. Strictly speaking, regulation of gene expression pertains to transcription of the gene; subsequent events are excluded. The following section deals exclusively with gene transcription and its regulation.

2.1. Structure of a Hypothetical Gene and Its Transcription

The structure of a hypothetical gene is shown in diagrammatic fashion in Fig. A1-2. The coding region, shown with three introns, is flanked by 5' upstream and 3' downstream regions. The 5' upstream region, the promoter region, contains the TATA box, which is important for the correct initiation of transcription of the gene.

2.2. Basal Transcription–Initiation Complex and Activators

For a study of gene transcription, the coding region is of little interest. Of more interest are the 5' promoter and 3' flanking regions, especially the promoter region. For a gene to be transcribed, some proteins localized in the nucleus and known as transcription-

factors must recognize and bind to certain nucleotide sequences in the promoter—the so-called *cis* sequences; *cis* refers to action on nucleotide sequences (or elements) “in line” with the coding sequence. In contrast, *trans* refers to action through an intermediate (RNA or protein) and thus does not have to be directly linked to the affected gene; *trans* factors (or elements) include transcription factors, enhancers, and inhibitors; they all act in *trans*, i.e., they affect the function of genes that they are not directly related to.

DNA–protein interactions are crucial to gene transcription. Short stretches of nucleotides (each stretch usually no more than 20 nucleotides) in the DNA double helix cause specific surface patterns of H-bond donors, H-bond acceptors, and hydrophobic patches (Fig. A1-3). These patterns, which are determined by the order of nucleotides in a stretch of DNA, are

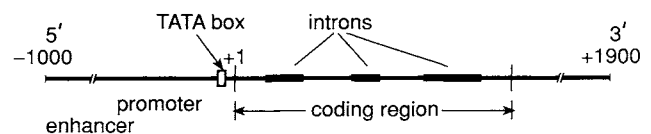


FIGURE A1-2 Structure of a hypothetical gene. The coding region is shown with three introns. The 5' region, upstream of the coding region, is known as the promoter region; it extends into the enhancer region, although a precise delimitation between the two regions is not possible. The promoter region contains the TATA box. Base pairs are numbered negatively upstream from the transcription start site. The figure is not to scale and -1000 and +1900 are hypothetical numbers.

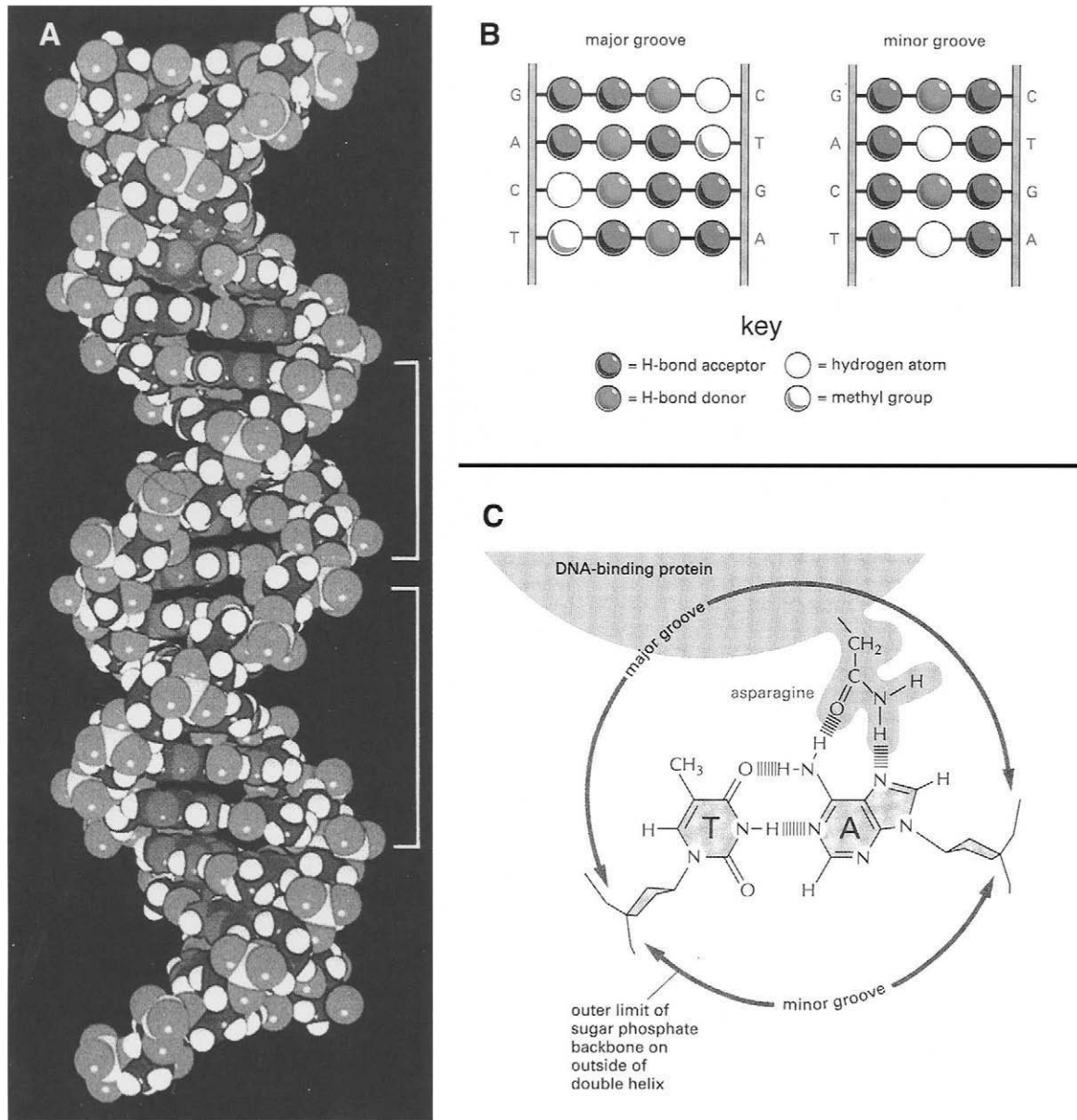


FIGURE A1-3 DNA structure and binding of regulatory proteins. (A) DNA double helix showing major (larger bracket) and minor (smaller bracket) grooves. (B) DNA recognition patterns. The edge of each base pair, seen here looking directly at the major or minor groove, contains a distinctive pattern of hydrogen bond donors, hydrogen bond acceptors, and methyl groups. From the major groove, each of the four base-pair configurations projects a unique pattern of features. For the minor groove, however, the patterns are similar for G-C and C-G, as well as for A-T and T-A. (C) Binding of a protein to the major groove in DNA. Only a single typical contact is shown; in an actual interaction, there would be 10–20 such contacts involving different amino acids and different bases, each contributing to the binding energy of the protein–DNA interaction. Reprinted with permission from Alberts *et al.* (1994), © Garland Publishing, Inc.

recognized by specific sequences of amino acids in proteins. Such recognition occurs at the major groove, from the outside, without necessarily opening the double helix. In any protein–DNA sequence interaction, many such contacts are made, which though individually weak, together constitute a very tight and specific fit.

Most eukaryotic genes contain a *cis* sequence, the TATA box, a sequence of nucleotides rich in A and T, which is recognized by a specific and highly conserved TATA-binding protein (TBP). The TBP sits atop the DNA double helix like a saddle, and in association with several other proteins [TBP-associated factors (TAFs), sometimes referred to as the transcription

factor complex IID or (TFIID)] and RNA polymerase II (POL II) forms the initiation complex, which ensures that transcription of the gene in question by POL II starts at the correct initiation site and progresses to completion (Fig. A1-4).

Binding of the initiation complex to the TATA box allows a certain basal level of gene transcription. Many genes also contain other *cis* sequences, known as "enhancer" elements, which occur "at a distance" further upstream from the promoter or in the 3' region downstream of the coding region. Enhancer elements are bound by other transcription factors, known as "activator" proteins. Many of the proteins in the initiation complex have been shown to interact with activator proteins *in vitro*. Hence, it is thought that binding of the transcription factors to DNA induces a conformational change in the DNA such that activator proteins and the proteins in the initiation complex are brought in close proximity to each other (see Fig. A1-4).

2.3. Gene-Specific Transcription

Basal factors in the initiation complex that bind to the TATA box are responsible for the accurate and efficient transcription of all genes. They can be considered general transcription factors. In most cases, *cis* sequences and *trans* factors, unique to a particular inducing signal and/or unique to a specific tissue or developmental pathway, are also required. *In the following treatment, the example of a hormone as an inducing signal is used, although any other environmental signal, such as light or temperature, could be used as well.* A study of hormone-induced gene expression involves a search for *cis*

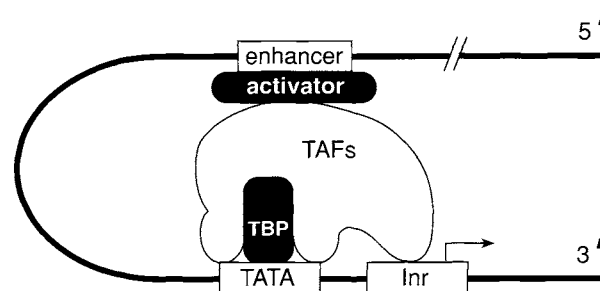


FIGURE A1-4 Organization of the transcription initiation complex. The initiation complex is a large protein machine responsible for the correct initiation of transcription. It consists of the TATA-binding protein (TBP), as many as 20 associated proteins, and transcription-associated factors (TAFs, also referred to as TFIID), which are shown here as a group. Some of these proteins make independent contacts with the promoter sequences. The initiator element (Inr) is a sequence of nucleotides that span the transcription start site. The activator protein binds to the enhancer and also interacts with a TAF. The occurrence of enhancer elements at a distance allows "looping" of the DNA. Positions of the enhancer, TATA element, and Inr are indicated in boxes. RNA polymerase II is not shown. Modified from Sachs and Buratowski (1997) with permission from Elsevier Science.

sequences and *trans* factors that are unique to a particular hormone and/or tissue.

2.3.1. Identification of *cis* Sequences

In order to identify the *cis* sequences important for a particular hormone, the 5' upstream regions, may be up to -1000 bases, in a number of genes that are known to be induced by that hormone are sequenced. Nucleotide sequences that are common, or "conserved," among different genes are identified. Each such sequence is usually less than 20 nucleotides in length (Table A1-1).

Table A1-1 Highly Conserved Barley α -Amylase Gene Promoter Sequences^{a,b}

Clone		Pyrimidine box		-130 sequences	-105 sequences
Low pI					
Amy32b	-134	TTGCACCTTTTCTCGTA	-120	GTAACAGAGTCTGG	TATCCATGC
gKAmy 155.3	-229INV	CATTGCCTTTTGCTTTT	-133	ATAACAGAGGCCGG	TACCCATGC
High pI					
gKAmy141.117	-170	ATTTCGCTTTTGAGCTC	-140	ATAACAAACTCCGGCCGACATATCCATCG	
Amy6-4	-173	AATCGCCTTTTGAGCTC	-143	ATAACAAACTCCGGCCGACATATCCACTG	
Amy6-4	-437	TAAACCTTTTGGGGTT			
Amy46	-170	AGTCGCCTTTTGAGCTC	-140	ATAACAAACTCCGGCCGACATATCCATCG	
gRAmy152	-175	AATCGCCTTTTGAGCTC	-140	ATAACAAACTCCGGCTGACATATCCACTG	
gRAmy56	-171	AGTCGCCTTTTGAGCTC	-141	ATAACAAACTCCGGCCGACATATCCATCG	
pHv19	-174	AATCGCCTTTTGAGCTC	-144	ATAACAAACTCCGGCCGACATATCCACTG	

^aFrom Jones and Jacobsen (1991).

^bSequences are aligned for better comparison, and common sequences are in bold letters.

2.3.1.1. Functional Characterization of *cis* Sequences

Identification of conserved *cis* sequences is only the first step. In order to show that any of these conserved sequences has any functional significance, it must be shown that it is indeed important for the hormone-induced transcription of the gene in question. This is made possible by making fusion constructs of the promoter region of the gene with the coding sequence of a reporter gene (Fig. A1-5). There are many reporter genes. Most are nonplant in origin; their encoded products “report” their presence by some vivid color reaction or luminiscence. One of the very common reporter genes used in plant molecular work is the bacterial β -glucuronidase (*UidA* or *GUS*) gene. It is particularly useful because it does not naturally occur in plants and encodes an enzyme that gives a distinct blue color when presented with its substrate. Other reporter genes include the *LUCIFERASE* (*LUC*) gene from firefly, chloramphenicol acetyltransferase (*CAT*) gene from bacteria, and the gene for green fluorescent protein (*GFP*) from the jelly fish, *Aequorea victoria*. The *GFP* gene encodes a protein that has the property to emit green fluorescence when excited by light of higher energy (UV blue). It can be expressed in most cells, with only minor modifications, and what is most useful can be used to report *in vivo*, or in living cells, whereas other reporters usually involve killed (or fixed) and sectioned material.

Deletion Analysis Fusion constructs are made in a series, with lesser and lesser parts of the original promoter (Fig. A1-6). The series is referred to as a “deletion series” and the assay deletion analysis. These constructs can then be introduced in a suitable tissue or plant, either transiently using any of the different methods of transformation [e.g., bombardment with a particle gun, polyethylene glycol (PEG)-mediated transfer, electroporation] or stably using the *Agrobacterium*-mediated transformation system (for these methods, see Appendix 2). For most routine work, transient expression in protoplasts, or single cells, is used, and the expression of the reporter gene is monitored after +/– hormone treatments. The deletion constructs

identify the minimal length of the 5′ upstream sequence that is necessary for hormone-induced expression of the gene.

Fusion Constructs Can Be Modified in Several Ways The part of the promoter identified from analysis can be used to provide information on which specific *cis* sequences are important for induction by a hormone. For this work, usually a minimal promoter (a short sequence containing the TATA box and the initiator site) from an unrelated organism (a heterologous promoter) is substituted and fused at its 5′ end to the promoter fragment(s) of interest and at its 3′ end to the reporter gene. The minimal promoter may come from different sources. One of the minimal promoters commonly used is the 35S cauliflower mosaic virus (CaMV) promoter. It is a strong promoter, which is expressed constitutively, i.e., without the need for any transcriptional factor(s) (see Section 2.4) other than those in the basal initiation complex. Other constitutive promoters come from genes that are nearly universally expressed in eukaryotic cells, such as *ACTIN* or *UBIQUITIN* genes. A heterologous promoter from a different gene of the same plant may also be used.

i. Effect of orientation and number of copies of a sequence. Constructs with reversed orientation or multiple copies of a *cis* sequence believed to be important can be fused to a strong but minimal promoter and to a reporter gene and introduced to see the effect of orientation or number of copies on gene expression (Fig. A1-7A).

ii. Effect of unrelated sequences. Two unrelated *cis* sequences may be inserted along with the promoter and reporter gene construct to see whether the two sequences are complementary to each other and act in concert or are antagonistic to each other.

iii. Effect of mutagenesis. A sequence believed to be important can be mutagenized in a site-specific manner and inserted along with the promoter and reporter gene construct. If the mutation causes a reduction or loss in expression of the reporter gene, it means that the sequence is important.

In a modification known as linker scan analysis, a noncoding sequence (an intron) is used to “link” the minimal promoter to the reporter gene and mutations are introduced in the promoter segment of interest, every *x* number of bases (Fig. A1-7B).

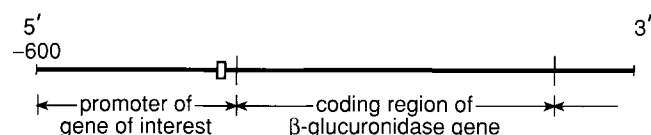


FIGURE A1-5 Diagrammatic illustration of a fusion construct. The promoter region of a gene, say up to –600bp, is fused to the coding region of a reporter gene (e.g., *GUS*) and a suitable 3′ end is added for proper transcription termination and polyadenylation of the message.

2.3.1.2. Response Elements

The analysis of *cis* sequences has led to the concept that there are specific *cis* elements, which are necessary for the induction of genes by a particular

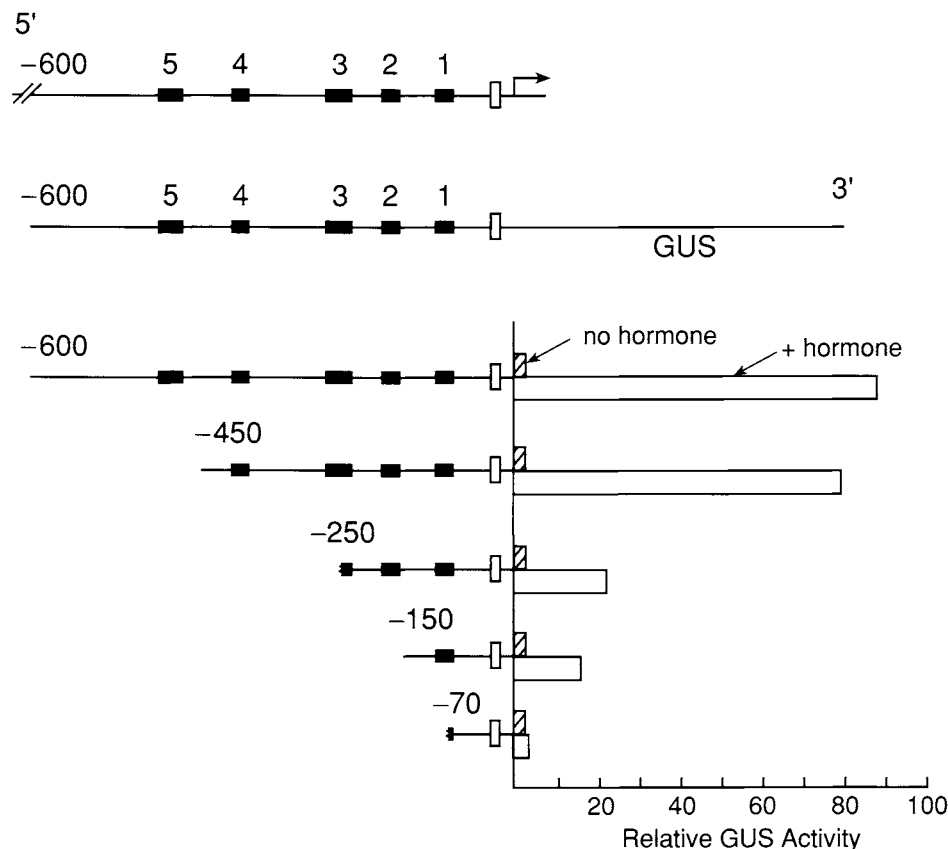


FIGURE A1-6 Deletion analysis of a promoter. The promoter is shown with six *cis*-sequences — 1–5 plus the TATA box shown as open vertical rectangle. A series of fusion constructs using different lengths of the promoter and coding sequence of the reporter gene, *GUS*, are prepared. These constructs are introduced in a suitable vector for transient expression after +/– hormone treatment. Relative *GUS* levels, +/– hormone, are shown in histograms opposite the constructs. The histograms indicate that the *cis*-sequences 1 and 4 are important for *GUS* expression and, by inference, hormonal action.

hormone or environmental signal. Many such elements, known as “response elements” or “boxes,” have been identified. For example, a common *cis* element known as the G box is found in promoters of many genes that are regulated by environmental signals, such as visible light, UV, and anaerobic conditions. Other boxes specific to drought, cold, heat shock, or other signals are known, as are response elements to many hormones. Single or multiple copies of these response elements (or promoter segments containing them), linked to a minimal promoter plus a reporter gene, confer inducibility by the appropriate signal in *in vitro* assays. However, in nature and *in planta*, most likely, these response elements do not act alone, but rather act in concert with one or two other *cis* elements. Such *cis* elements may be described as coupling elements because they couple hormone (or an environmental signal) inducibility to the response element. The combination of a response element and one (or more) other

cis element that provides the minimal promoter the ability to confer full expression of a gene is referred to as the response complex. A response complex for a UV-induced gene is shown in Fig. A1-8. Response complexes for other hormones are known also.

The combination of two or more *cis* elements provides an additional level of control over gene transcription by a specific signal. Moreover, it is possible that the various *cis* elements act as modules, which are interchangeable, thus providing a greater flexibility in the regulation of gene transcription. For instance, different modular combinations may be used for tissue and/or organ specificity in gene transcription induced by the same signal.

2.3.2 Gene-Specific Transcription Factors

General transcription factors comprising the basal initiation complex (see Section 2.2) are common to all

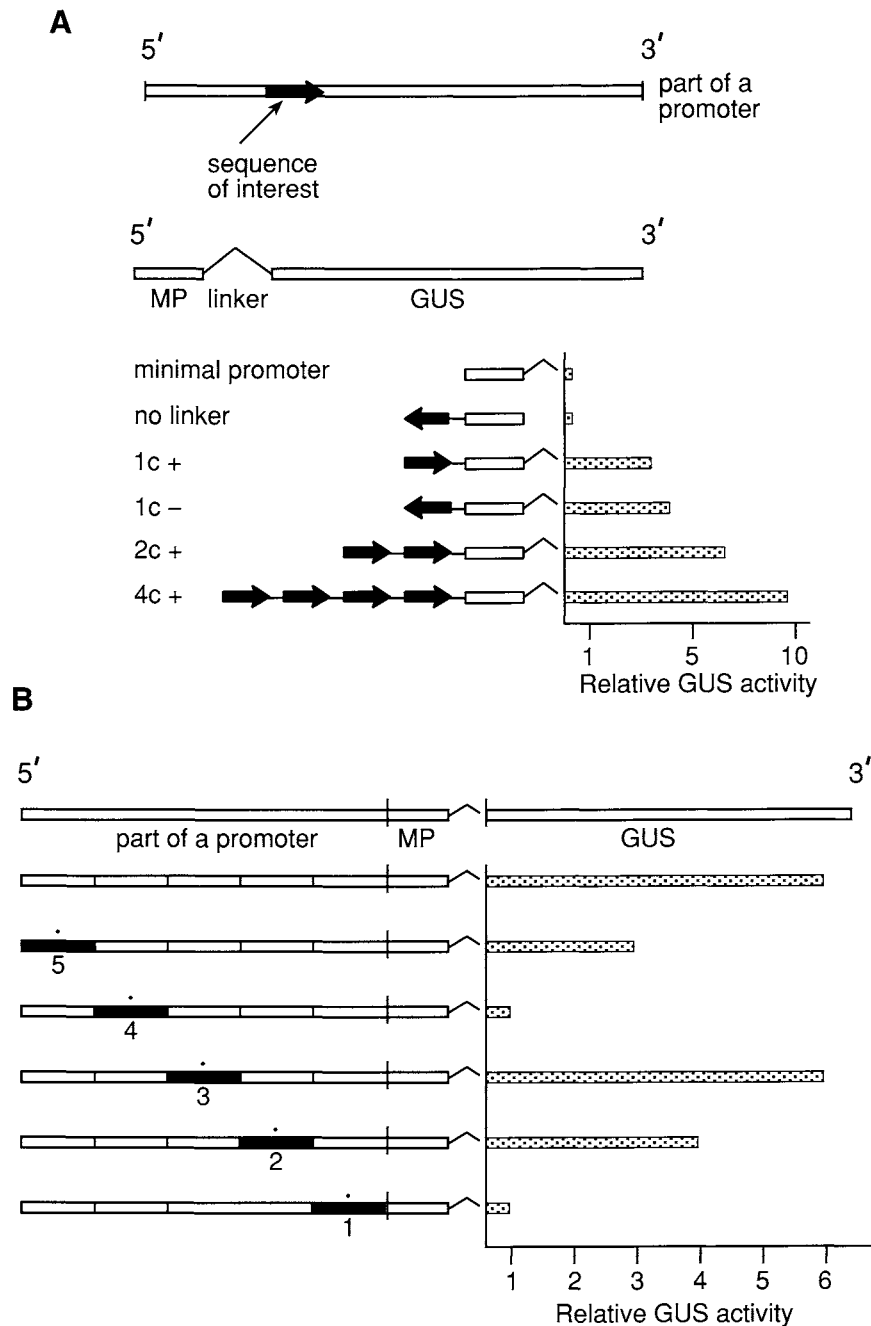


FIGURE A1-7 Effects of orientation, number of copies, and mutagenesis in a promoter segment on reporter gene expression. (A) A segment from the promoter of a gene, in +/- orientation and one to four copies (c), is fused to a heterologous minimal promoter (MP). The minimal promoter is linked *via* an intron fragment (thin black angled line) to the coding sequence of the β -glucuronidase gene for transient expression in a suitable vector. Relative GUS activity is shown in histograms opposite the constructs. (B) Linker scan analysis of a part of the promoter. Mutations were introduced at intervals of 10 bases in the above promoter fragment, and their effect on GUS expression was monitored. Mutations in segments 1 and 4 severely reduced the reporter gene expression; hence, these sequences are important for induction by the hormone.

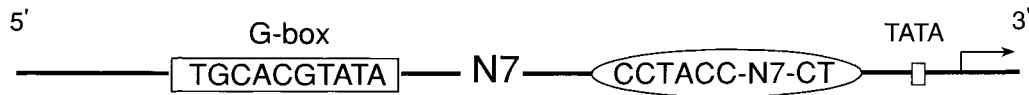


FIGURE A1-8 Response complex for a UV-induced gene. The rectangular box represents a response element, and the elliptical box is a coupling element. The two are separated by a sequence of seven nucleotides (N7). Modified from Shen and Ho (1997).

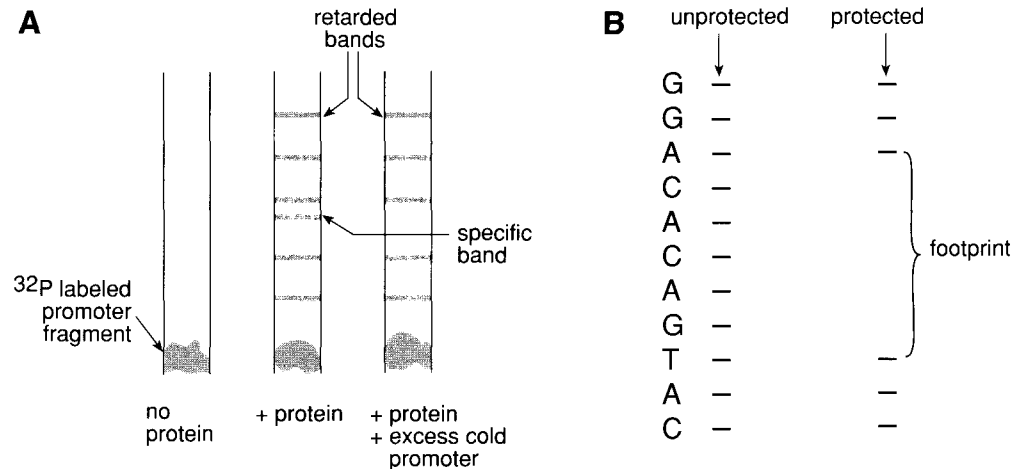


FIGURE A1-9 Gel retardation and footprinting assays. (A) Nuclear proteins are extracted and incubated with a ^{32}P -labeled fragment of the promoter, usually a 200 to 300-bp segment carrying a *cis* sequence(s) that is believed to be important. After a suitable incubation period, the protein–DNA mixture is run on agarose gels and the label is visualized by a phosphorimager or by exposure on X-ray film. In the absence of protein, all the label ends up at the bottom of the gel (left). In the presence of nuclear proteins, some proteins may bind to the labeled promoter fragments and retard their flow through the gel; thus, they appear as “retarded” bands (middle). To minimize nonspecific binding of proteins to the promoter fragment, an excess of noncompeting, heterologous DNA may be added to the incubation mixture. The retarded bands are checked for specificity of binding by adding an excess of unlabeled promoter segment (100–400 \times) to the incubation mixture, which reduces the intensity of labeling or eliminates the retarded band (right). (B) A region of DNA that is bound by a protein is protected against digestion by endonucleases, such as DNase I. Thus, after protein binding, the promoter fragment can be hydrolyzed and sequenced. A comparison of the two sequences, one with and one without protein binding, identifies the nucleotide sequences that were protected by the protein and gives what is described as a “footprint.”

genes, but they are of little interest for gene-specific transcription. Instead, we look for transcription factors that bind to *cis* elements in the promoter of a gene, or to a promoter of a gene in a tissue-specific manner, i.e., they are unique to the expression of the gene or expression of a gene in a particular tissue. Although general transcription factors are present in abundance, gene- and tissue-specific transcription factors usually occur in much smaller quantities.

2.3.2.1. Gel Retardation and Footprinting Assays

Transcription factors are isolated using DNA–protein interactions, which are highly sequence specific (see Section 2.2). Nuclear proteins are isolated, purified as far as possible, and incubated with a ^{32}P (or otherwise)-labeled segment of the promoter, which previous analysis has shown to be important. The mixture is

electrophoresed on a gel, and retarded bands are analyzed for specificity of binding (Fig. A1-9A). That this binding occurs in a specific region of the promoter is shown by footprinting assays (Fig. A1-9B). The footprint(s) helps in the identification and conformation of a conserved sequence as a response element.

Nuclear proteins that bind the promoter fragment in a gene- and tissue-specific manner can be isolated and partially sequenced. That amino acid sequence can be used to produce oligonucleotide probes, which can then be used to screen a cDNA library. Isolation of a cDNA clone for a transcription factor can lead to isolation of its gene, and its expression in a suitable vector can be used to obtain large amounts of the protein or confirm its function by functional complementation (see Box 2-3 in Chapter 2). By providing the means to produce large amounts of protein, it can also

facilitate the preparation of an antibody against the protein.

If care is taken in extraction and processing of DNA from a particular cell type or tissue, nuclear proteins bound to *cis* sequences can be visualized by what is known as *in vivo* footprinting. *In vivo* footprinting can indicate differences in protein-binding patterns between distinct signals or different parts/tissues. The identity of proteins bound to DNA remains unknown in these assays and is determined by *in vitro* footprinting.

2.3.2.2. Transcription Factors Have Conserved Domains and Are Grouped into Several Families

Transcription factors are proteins that are synthesized in the cytoplasm but are located in the nucleus. Hence, they carry nuclear localization signals (NLS), a sequence of amino acids that targets them to the nucleus after posttranslational modifications in the endoplasmic reticulum–Golgi complex. They also show several conserved domains. Because they bind to DNA, they carry a sequence of amino acids, usually basic amino acids, that bind to the major or minor groove in the DNA double helix — the DNA-binding

domain. Many of them are active in DNA-binding as homo- or heterodimers. Thus, they carry, in addition to the DNA-binding domain, specific sequences that are involved in binding to other proteins — protein–protein interactions. Dimerization of these proteins increases the tightness of binding to DNA; it also provides a greater specificity of binding because two heterodimers recognize different sequences in DNA. Many transcription factors serve to activate transcription; they carry yet another domain, the activator domain, that in ways still unknown interacts with the basal initiation complex or other transcription factors to activate transcription.

Based on their characteristic structural DNA-binding motifs, several families of transcriptional factor proteins have been identified in prokaryotes and eukaryotes. Outside of the DNA-binding motif, which sometimes combines the function of dimerization, the protein structure varies considerably within the same family. Thus, transcription factors belonging to the same family can be involved in widely different functions. Box A1-1 describes characteristics of the more prevalent families.

BOX A1-1 TRANSCRIPTION FACTORS

SEVERAL FAMILIES OF TRANSCRIPTION factors based on their DNA-binding motifs are known from plants and animals. Characteristics of the more common families are described.

1. Helix-turn-helix containing proteins. The helix-turn-helix motif is a simple DNA-binding motif. Two α helices are connected by a short extended chain of amino acids, which constitutes the “turn” and holds the two helices at a constant angle. The C terminus α helix serves to recognize the DNA sequence and bind to the major groove (Fig. A1-10A). Because the structure of the protein outside the DNA-binding motif is different for different proteins, each protein carrying the motif can present the recognition helix to DNA in a unique way. These proteins bind as symmetric dimers to DNA sequences that are composed of two very similar “half-sites,” which are also arranged symmetrically. The helix-turn-helix motif also occurs in other types of transcription factors.

2. Basic-region leucine zipper (b-ZIP) proteins. A leucine zipper protein is composed of two α helices. One helix is rich in basic amino acids, serves as the recognition helix and binds to the major groove in DNA. The other helix carries a sequence of three to six repeats in which leucine occurs at regular intervals. It presents hydrophobic side chains on one side, which allows binding to another monomer with similar hydrophobic side. Proteins that contain a leucine zipper motif form homo- or heterodimers, which look like an inverted ‘Y’, with the arms of the Y holding DNA (Fig. A1-10Ba). If heterodimers, they recognize different DNA segments, thus increasing the repertoire of DNA-binding specificities of these proteins. Two types of monomers can combine in three different combinations (Fig. A1-10Bb).

3. Basic region helix-loop-helix (b-HLH) proteins. In bHLH proteins, a short α -helix is connected by a loop to a second, longer α -helix. These proteins also form homo- or heterodimers. The longer α -helix in each monomer has a sequence of basic amino acids and binds to DNA, whereas the shorter helix binds to another monomer. Several HLH proteins are truncated and lack the extension of the α -helix to bind to

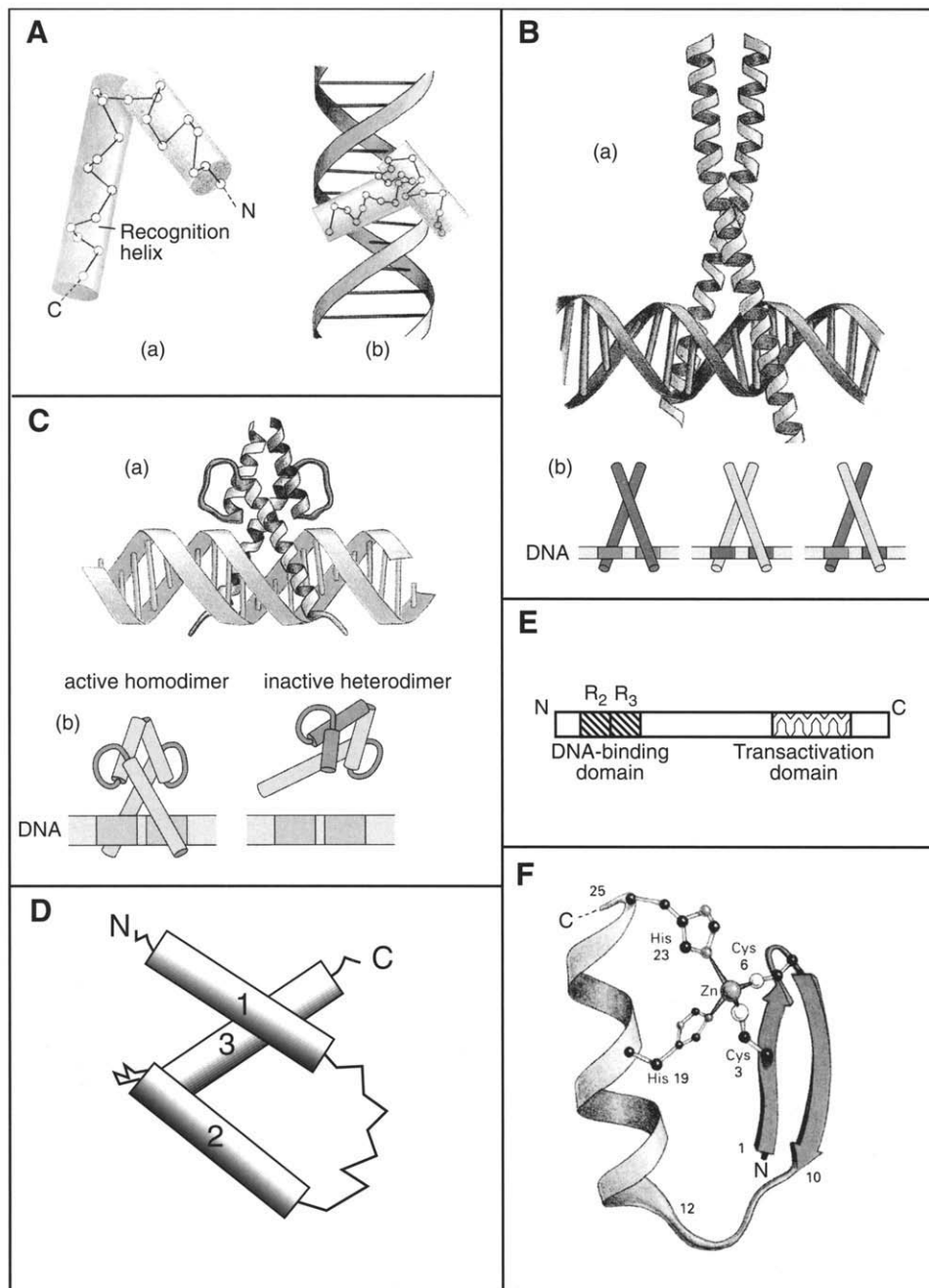


FIGURE A1-10 Transcription factors. Panels A, B, C, and F from Albertis *et al.* (1994).

DNA (Fig. A1-10C). Thus they can form heterodimers only with full-length monomers, but the binding strength is only half. Heterodimerization provides a useful control mechanism enabling a cell to inactivate specific gene regulatory proteins.

4. Homeodomain-containing proteins (HD proteins). These proteins carry a conserved domain of about 60 amino acids known as homeodomain. The homeodomain contains three α -helices: helices 1 and 2 are separated by a loop and pack into an antiparallel arrangement, whereas helices 2 and 3 form α -helix-turn-helix unit packed at an angle of about 120° (Fig. A1-10D). Helix 3 is the recognition helix and binds to the

DNA sequence. The helix-turn-helix motif is embedded in the homeodomain, and the amino acids adjacent to the motif ensure that the recognition helix is presented to DNA in the same basic manner. Homeodomain proteins play an important role in pattern specification during development in both plants and animals. They are a large family of transcription factors, which is divided into many groups and subgroups based on sequence comparisons of homeodomains and adjacent regions. HD proteins combining a homeodomain with other motifs, such as a leucine zipper (HD-bZIP), are also known.

5. MYB proteins. MYB proteins have a DNA-binding domain, the Myb domain, which usually has two or three, tandem, imperfect repeats (R_1 , R_2 , R_3) of 51–53 amino acid residues, each containing tryptophan residues at conserved positions. MYB proteins, designated after the oncogenic component of myoblastoma virus, in chicken and humans have three repeats with three α helices in each. The third α helix in R_3 is thought to bind to the major groove in DNA. Outside of the conserved repeats, there is little similarity between different MYB proteins. Myb domains in plants generally contain only two repeats: R_2 and R_3 (Fig. A1-10E). The MYB family is well represented in plants (> 100 members in *Arabidopsis*, ~40 in petunia, many in maize) and regulates a vast array of genes involved in diverse functions. One of the major groups of MYB proteins in plants regulates structural genes encoding enzymes in phenylpropanoid pathway, with the pathway leading to the synthesis of flavonoids and lignin.

6. MADS box-containing proteins. The name derives from the initials of four initially identified members: yeast MCM1, *Arabidopsis* AGAMOUS and *Antirrhinum* DEFICIENS, and the human serum response factor (SRF). The MADS box motif is composed of 56 amino acids; it can be divided into a basic hydrophilic region, which is involved in DNA recognition, and an acidic hydrophobic region. MADS box proteins are especially important in flower development in plants.

7. Zinc finger-containing proteins. There are several types of DNA-binding proteins with a zinc finger motif. These proteins have a stretch of about 25 amino acids, about half of which form an α helix, whereas the other half forms two antiparallel β sheets; one of the β sheets together with the α helix holds a zinc atom via Cys and His residues (Fig. A1-10F). Other proteins may have two α helices holding up the zinc atom. In both types, an α helix is used as the recognition helix for the major groove.

8. Other transcription factors. In addition to the types just given several other families of transcription factors, such as the APETALA2 (AP2) family, GRAS family, and those with a $\beta\alpha\alpha$ motif in the DNA-binding domain, have been reported and appear to be unique to plants. Relevant details about these transcription factors are provided at appropriate places.

Some transcription factors do not have a DNA-binding domain, yet are able to modulate the activities of other transcription factors *via* protein–protein interactions. These transcription factors are sometimes called transcription activators, a term more commonly used to denote proteins that bind to enhancer elements (see Section 2.2). These transcription factors may act independently of a hormonal signal to activate a gene in a tissue- and/or organ-specific manner; in other cases, they may act in concert with a hormonal signal to activate a specific gene.

2.4. Transcription of Some Genes Requires Prior Protein Synthesis

The transcription of some genes is inhibited if protein synthesis inhibitors, such as cycloheximide, are included in the medium (Fig. A1-11). Basically, this means that the transcription of that gene requires the prior synthesis of a protein. In contrast, if the expres-

sion of a gene is not inhibited by cycloheximide, then that gene is considered a primary response gene and

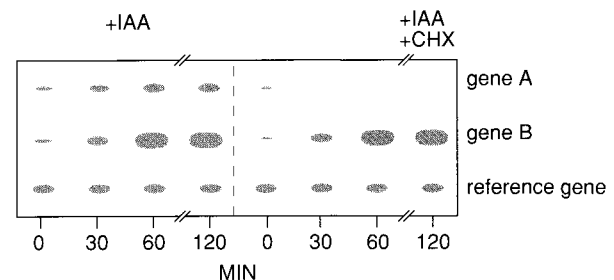


FIGURE A1-11 Effect of cycloheximide (CHX) on accumulation of mRNAs of two genes regulated by indoleacetic acid (IAA). The expression of gene A is inhibited by cycloheximide, as shown by lack of its mRNA in the presence of cycloheximide, whereas that of gene B is not. Expression of gene A requires the prior synthesis of another gene product. Gene B is a primary response gene. A reference gene serves as a control. Its expression is unaffected by either IAA or cycloheximide. Reprinted with permission from Alberts *et al.* (1994), © Garland Publishing, Inc.

its induction does not require the prior synthesis of any other protein. As expected, many of the primary response genes encode proteins that have turned out to be transcription factors, which are essential for the transcriptional regulation of other genes.

2.5. Summary of Gene Transcription

In summary, gene transcription is a complicated process that involves, in addition to basal transcription factors and RNA polymerase II, binding of many other nuclear proteins to specific sequences in the promoter and enhancer regions of a gene. The sequences are conserved among different genes regulated by the same signal, but variability in regulation is provided by combinations of two, three, or more sequences acting in concert in a modular fashion. The modular nature of *cis* elements is paralleled by a diversity of gene-specific transcription factors, which may also form homo- or heterodimers. The combinations of *cis* elements and transcription factors provide a wealth of diversity, which is needed for the transcription of different genes, and of the same gene by a variety of signals in a tissue- and/or organ-specific manner. **Combinatorial control**, in which combinations, rather than an individual protein, control a cellular process, is one of the most important processes in eukaryotic gene control. It is accomplished to a large extent by combinations of *cis* elements and heterodimeric regulatory proteins.

3. *IN VITRO* TRANSCRIPTION

One of the impediments in the isolation of proteins that interact with genes, or in study of gene induction by a stimulus (e.g., a hormone), has been the lack of an *in vitro* transcription system, a system where a gene can be transcribed in a cell-free manner. The availability of such a system could be useful, for instance, in the isolation of receptor proteins for specific hormones. Run-on transcription assays by nuclei isolated from cells/tissues that have been preinduced by a stimulus have been available for some time, but those assays still represent a complex system (see Fig. A3-14A). Several laboratories have been actively pursuing the development of an *in vitro* transcription system since the early 1990s. To develop such a system, cell proteins need to be obtained in as natural and undegraded state as possible. Usually, cells in suspension culture are harvested at defined times and a cell extract is prepared, with care being taken to prevent actions of various proteases and nucleases, which are released when cells are homogenized. An enriched protein fraction is prepared by the

gradual precipitation of proteins with an increasing concentration of ammonium sulfate, followed by removal of salt by dialysis. This protein fraction contains all the various regulatory proteins of the initiation complex and enzymes essential for correct initiation, elongation, and termination of primary transcripts. For an assay, the protein extract, a DNA template, and all four nucleotide triphosphates in a suitable transcription buffer are required. To test correct initiation and termination, the reaction is stopped at defined times, and mRNAs are separated according to size. Because the length of the template DNA is known, the RNA transcripts can be matched against the predicted length to check whether transcription went to completion. A simplified protocol is shown in Fig. A1-12B. To date, greater success has been obtained with the circular plasmid DNA template rather than a linear template, but with time, more progress is expected.

4. GENETIC TECHNIQUES

Use of mutants in the analysis of developmental patterns, elucidation of metabolic pathways, or signaling is a powerful tool for three reasons. (i) Mutations work in a natural setting; there is no need for external chemicals or treatments; (ii) mutant phenotypes provide some preliminary clues as to the character(s) affected; and (iii) mutant alleles provide a path to cloning the wild-type gene and thus assignment of function(s) to the encoded protein. However, mutational analysis has limitations as well. Not all genes can be uncovered by mutagenesis. For example, genes that share overlapping functions with other genes and are functionally redundant are difficult to identify because mutations in them do not yield an easily recognizable phenotype.

4.1. Mutagenesis and Screens

Mutants occur in natural populations, but for most experimental work, mutagenesis is carried out in the laboratory, using chemical, physical, or genetic means. Seeds, spores, or seedlings may be mutagenized chemically by treating them with ethylmethanesulfonate (EMS), diepoxybutane, or physically by subjecting them to high-energy radiation (e.g., X rays, γ rays, fast neutrons). Chemical mutagenesis usually results in single nucleotide changes (point mutations), whereas high-energy radiation usually causes deletions of large sequences of nucleotides and chromosome breakage. Mutagenesis is also carried out by inserting foreign DNA into random positions in the plant genome. For example, plants may be transformed using disarmed

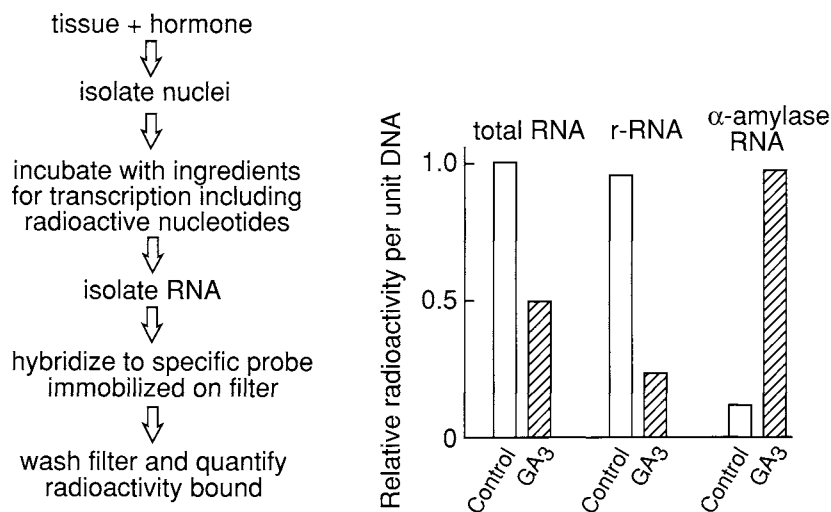
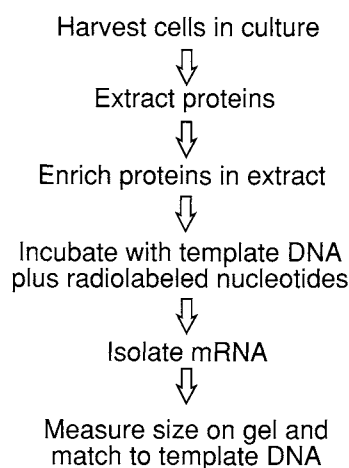
A**B**

FIGURE A1-12 Protocol for run-on transcription by isolated nuclei (A) and *in vitro* transcription in a cell-free system (B). (A) Protocol and accumulation of GA-specific transcripts (α -amylase RNA) by nuclei isolated from barley aleurone protoplasts treated with buffer only (control) or with buffer plus GA₃. Transcript accumulation drops off after some time. Values presented are after 1 h incubation. (B) A simplified protocol for *in vitro* transcription.

T-DNA from Ti plasmid in *Agrobacterium* (see Appendix 2) or a transposon, such as maize *Ac/Ds*. The advantage of insertional mutagenesis is that the T-DNA or the transposon serves to “tag” the gene in which it is inserted, allowing easier isolation of the mutated gene and its cloning (see Box A1-2).

Mutations occur randomly, and screening and selection of mutants for desired traits are important first steps. A multitude of screens have been developed since the early 1990s. Commonly, screens are based

on a modified phenotype, i.e., a phenotype that is distinct and different from the wild type. Phenotype includes not only morphological, but also physiological and biochemical characteristics. For instance, for selection of mutants that have a reduced sensitivity to a hormone (or some other signal), involves growing mutagenized seeds in the presence of the hormone and screening for a phenotype that shows a lack of the expected hormonal response (resistance or insensitivity to a hormone). Alternatively, mutagenized

seedlings may be grown in the absence or a suboptimal concentration of the hormone and screened for a phenotype that appears as if it has been supplied with a saturating concentration of the hormone (constitutive response or enhanced sensitivity).

The selected seedlings are raised to maturity (the first generation of mutant plants is referred to as the M1 generation), flowers are typically self-pollinated, and seeds from each plant are collected separately or as pools of seeds from a number of plants (seed families). Seeds are germinated, and the progeny (M2 generation) are checked to ensure that the mutant phenotype is heritable. Seed families that contain mutant embryos/seedlings are worked further. Back-crosses to the wild type indicates whether the mutant allele is dominant or recessive. Some mutations are lethal, and the homozygous mutants do not survive past a certain stage. In such cases, heterozygous plants (with normal or wild phenotype) from each seed family are raised to maturity, selfed, and the presence of mutant embryos (M3) confirmed (Fig. A1-13). To determine which mutant characters are inherited together, seedlings with normal phenotype are raised to maturity, stamens are emasculated, cross-pollination is effected, and seedlings are scored for unlinked mutations.

4.1.1 Dominant vs Recessive Mutations

Recessive mutations are loss-of-function mutations and their interpretation is relatively straightforward. A loss-of-function mutation usually means that the encoded product has lost its function or has reduced function because of a mutation in its gene. Thus, if a recessive mutation activates a response, its wild-type

gene is thought to negatively regulate that phenotype. In contrast, dominant mutations, may be gain-of-function or loss-of-function mutations. Gain-of-function mutations alter the function of a protein to give enhanced/increased or new function, (e.g., by over-activity or ectopic expression). Dominant loss-of-function mutations can be caused by interference of the normal product by the mutant product. Such mutations are often referred to as dominant negative mutations. Specific techniques, such as mutation by "activation tagging," are used specifically to obtain gain-of-function mutations.

Activation tagging is a modification of T-DNA tagging. Multiple copies of enhancer elements from the strong constitutive promoter of cauliflower mosaic virus (CaMV) 35S are incorporated near the right border of a T-DNA. The construct, if inserted near a gene, may cause enhanced transcription of that gene, resulting in a dominant mutation. The new phenotype is recognized because of dominant mutation, and tagging allows cloning of the gene.

Mutagenesis, screening, genetic crossing, and characterization of mutant lines require plants that have a short generation time, can be easily crossed by the scientist, produce abundant seeds per plant, and, which at maturity, are still reasonably small, such that a large number of plants can be grown in the limited space of a laboratory or green house. *Arabidopsis thaliana* fulfills these conditions admirably: its small size and short generation time allow screening of thousands of seedlings very rapidly and, hence, it is one of the most commonly used species for molecular genetic work. Other plants, such as *Zea mays* (maize), *Oryza sativa* (rice), and *Lycopersicon esculentum* (tomato), although not so small nor with such a short generation time, nonetheless, produce abundant seeds and their genetics are well worked out because of their economic importance.

4.2. Isolation and Characterization of Genes

Several methods for cloning genes following mutational analysis are available. Two common methods are map-based cloning and gene tagging (see Box A1-2). Chemical mutagenesis, which results in single base changes, is usually followed by map-based cloning. Isolation of a mutated gene using this method within a reasonable time frame requires a plant with a relatively small genome size, a relatively small number of haploid chromosomes, and preferably one where enough genetic information is available on linkage groups and positions of marker genes in the genetic map. In these criteria again, *A. thaliana* is a remarkable

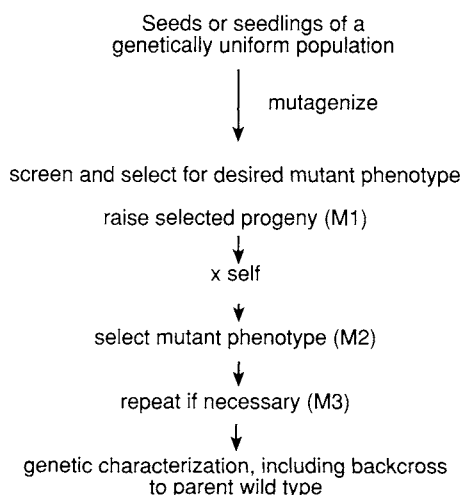


FIGURE A1-13 Protocol for creating and selecting mutant phenotypes.

plant. It has five haploid chromosomes; the haploid nuclear genome is only about 12×10^7 bp long, about 15 times that of *Escherichia coli*, and about 5 times that of fission yeast (*Saccharomyces cerevisiae*); there is a good deal of information on linked characters; and the entire genome has been mapped. Many other plants, such as maize, rice, and tomato, are also genetically very well characterized, although they have a bigger genome size and a longer generation time. For these plants, insertional mutagenesis or gene tagging is often used.

To isolate a gene, it is ideal to have a range of mutant alleles, from null to weak, such that the mutant phenotypes can provide some idea about the function of the gene. A series of mutant phenotypes that differ from the wild type to varying degrees would suggest that the wild-type gene product acts in a quantitative manner. In contrast, if all the mutant alleles give the same phenotype, then a threshold level of gene activity affects the biological response.

BOX A1-2 ISOLATION OF GENES USING MUTANTS AND VICE VERSA

HAVING A MUTANT AND the corresponding gene product is the ideal starting point for exploring how the gene acts in an organism. It is important to realize that both are needed. Having only the mutant or only the gene product is a severe limitation. A number of recent molecular tools that enable researchers to switch from mutant to gene and from gene to mutant are available and some are described.

1. From mutant to gene. Two basic strategies allow identification and cloning of a gene from a mutation. In both, additional analyses need to be made to verify the identity of the gene and to ensure the gene is the same as that defined by the mutation.

a. *Map-based cloning*. Map-based cloning is often used for mutations caused by chemical mutagens, which result in small changes in nucleotide sequences. To clone the gene, the mutation is genetically mapped relative to molecular markers (and to morphological markers if convenient). For mapping, a cross is made with a strain that carries nucleotide differences due to normal polymorphism between strains. The progeny can be allowed to self fertilize to produce the F₂ mapping population, which contains meiotic recombination between the chromosomes of the two parents. The gene of interest can be mapped by virtue of the fact that the mutation lies on a chromosomal segment derived from just one of the original parents. The position of the locus can be mapped on the basis of segregation with DNA markers. The DNA markers are slight sequence deviations between homologous chromosomes that can be easily visualized by techniques, such as polymerase chain reaction (PCR). Given a genetic map of molecular markers and a large segregating population, a mutation can be mapped to a chromosome interval encoding only one or a few mRNAs (Fig. A1-14).

b. *Gene tagging*. The mutation may be caused by the insertion of a piece of DNA, usually a disarmed T-DNA, or any other relatively short known sequence of DNA. When plants are transformed with exogenous DNA, the integration of this DNA in the nuclear genome may affect the expression of a gene located near the integration site; thus causing a mutant phenotype. A hybridization probe (that can be made for any known sequence) will detect the inserted DNA in the genome of the mutant plant, and the inserted DNA should be at the disrupted gene (Fig. A1-15). Although this is an idealized scenario, a piece of inserted DNA serves as the tag for a mutation-defined gene (hence, the term "gene tagging"). Compared to chemical mutagenesis, however, considerably more work is required to establish an insertional mutant collection.

Isolation of the gene tag on a particular restriction fragment will provide some of the flanking genomic DNA, which can be sequenced. The tag is often designed from a plasmid that is viable in *E. coli* for rapid rescue of the gene tag. The affected transcription unit may be identified by standard molecular biological techniques or by sequence database analysis in the case of *Arabidopsis*. Alternatively, a genomic library of the mutant may be generated, and the genomic fragment that hybridizes with the probe isolated and sequenced. Proof that the right transcription unit has been isolated is provided by isolating the wild-type gene and comparing the nucleotide sequences of the wild-type and several mutant alleles. Proof is also

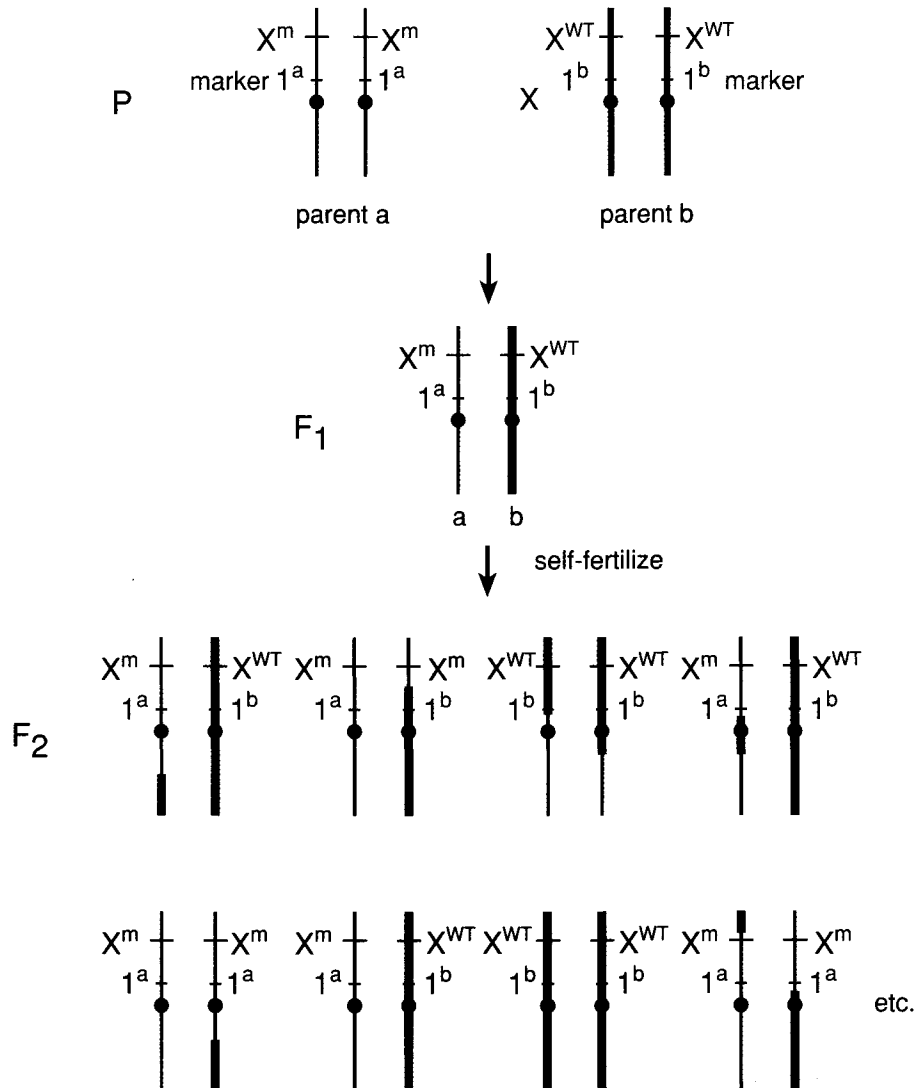


FIGURE A1-14 Map-based cloning. Lines/rectangles with black dots symbolize pairs of homologous chromosomes. The distance between a gene of interest (X) and a molecular marker (1) is determined by meiotic mapping. Briefly, an F1 plant is generated by crossing parents a and b, where parent a has the mutant allele of gene X (X^m) and parent b has the wild type allele X^{WT} . Because there is abundant sequence divergence between the two parents, numerous molecular markers can be visualized, which identify two different sequences at fixed chromosomal positions, for example, by generating PCR products of distinguishable length. Meiosis in the F1 individual will produce gametes that will either contain one of the parental marker combinations (X^m , 1^a or X^{WT} , 1^b) or, by crossing over, new recombinant marker combinations (e.g., X^m , 1^b ; X^{WT} , 1^a) that will give rise to the F2 mapping population. If a molecular marker (1) is very close to gene X, hardly any recombinant meiotic products will be produced. By contrast, if the marker is away from the gene of interest, or on another chromosome, parental and recombinant marker combinations will be produced at random frequencies. Using the frequency, standard calculations for meiotic mapping can be applied. Thus, given a sufficiently large segregating population, molecular markers extremely closely linked to a mutation-defined gene can be identified.

obtained by rescuing the mutant phenotype upon introduction of a wild-type copy of the suspected gene into the mutant ("transgenic complementation" or "transgenic rescue").

2. From gene to mutant. The reverse strategy, from gene to mutant, is also feasible. Because targeted mutagenesis, i.e., direct replacement of a wild-type copy by a mutant copy of a gene, does not work efficiently in higher plants, mutants of a given gene sequence are identified by "reverse genetic" screening of large populations of insertional mutants that are expected to contain insertions in virtually all genes. Since in this case the sequence of the gene of interest is known, primers to its sequence can be designed (Fig. A1-16). Briefly, a PCR reaction between a primer from the gene of interest and another one from the

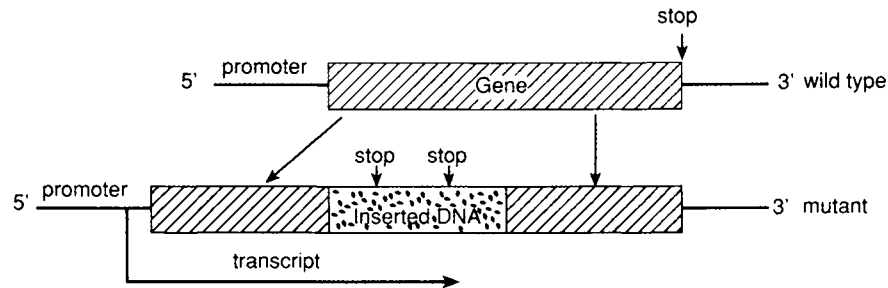


FIGURE A1-15 Gene tagging. A piece of inserted DNA has disrupted a gene (indicated by a transcript arrow) (lower line). The insertion can interfere with many aspects of gene expression and function. In this example, it prevents translation of a meaningful protein, which would have the further C-terminal amino acids from the site of the insertion. This may render the protein partially or totally nonfunctional. In the illustration above, the gene and inserted DNA reside on a single restriction fragment.

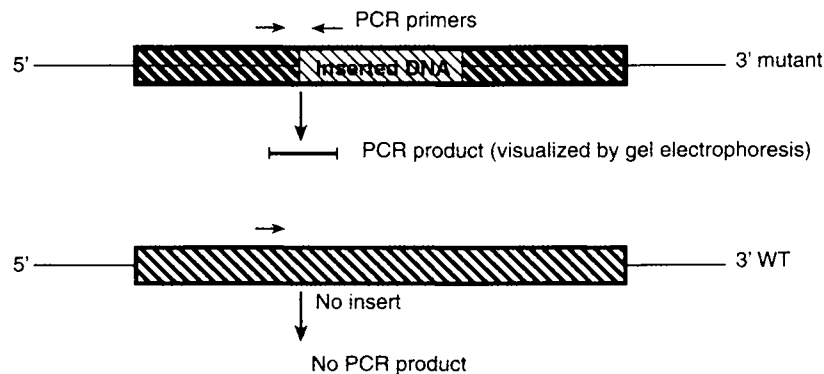


FIGURE A1-16 Reverse genetic screening of insertional mutations. Binding of a primer to the known sequence of the gene of interest (top row, left) and of another primer to the inserting DNA (right) will only produce a product if the insertion has occurred in the gene of interest.

common inserted DNA will only result in a PCR product if the two primers are located adjacent to each other (~1 kbp). Therefore, a successful PCR reaction indicates the integration of the insertion DNA into a gene of interest. Because the PCR reaction is extremely sensitive, the DNA of hundreds of insertional mutant lines can be pooled. Insertion in the gene of interest will remain visible as a PCR product. This line can subsequently be identified within a positive pool.

Box A1-2 is adapted from <http://www.botany.utoronto.ca/courses/bot340/BOT340,_molecular_tools.html>, courtesy of Thomas Berleth.

4.3. Transgression and Epistasis

Mutant phenotypes, while indicating that a gene affects a character (or characters), give little indication of the manner in which the character(s) is affected. For that the wild-type gene needs to be cloned (see Box A1-2) and a judgement made about the nature and possible function of the encoded protein. However, some conclusions can be made about the mutants and, by inference, their wild-type genes. If two mutations possess a similar phenotype, a technique known as transgression analysis is used to determine whether

the products of the two wild-type genes act in the same pathway or in separate pathways that converge. This is accomplished by crossing the two mutants. If the phenotype of the double mutant shows an additive phenotype, it is likely that the products act in separate pathways that converge; for mutants with distinct phenotypes, if one phenotype remains unaffected (i.e., one mutation completely masks the other), they likely occur in the same pathway.

If two genes affect different steps in the same biochemical pathway, it is of importance to know the

hierarchy of expression, i.e., the order in which they are expressed. Those expressed later are considered to be epistatic to those expressed earlier, and the phenomenon is called **epistasis** (strictly speaking, epistasis is any interaction between genes, such that the phenotypic expression of one gene depends on the genotype of the other. It does not matter which comes first in the pathway). Epistasis is determined by analysis of double mutants (Fig. A1-17).

4.4. Targeted Screens

Targeted screens serve specific purposes.

4.4.1. Suppressor and Enhancer Screens

A mutant line that has been selected may be mutagenized, in turn, and the resulting population screened for suppression of the mutant phenotype, i.e., for plants that look like the original wild type. Alternatively, it may be screened for an enhanced mutational phenotype. These screens are known as “suppressor” or “enhancer” screens, respectively. Suppressor mutants are similar in phenotype to the original, but this similarity results from suppression of the first mutation, not from a restoration of the original allele. Such suppression may occur by a mutation in the same gene—an intragenic suppressor mutation—or in a different gene—an extragenic suppressor mutation. Likewise, the enhancer mutant results from mutation in the same gene or at a new locus that augments the

effects of the earlier mutation. Products of extragenic suppressor or enhancer mutations act as *trans* factors to inhibit or enhance the expression of the affected gene (see Section 2.2). The cloning of their genes can be useful in elucidating the function of the original wild-type gene in a signal transduction pathway.

4.4.2. Gene-Specific Screens

If the response elements for a particular gene are known (see Section 2.3.1.2), it is possible to identify other genes that affect or modulate the expression of that gene. Constructs consisting of the conserved *cis* sequences + minimal promoter + reporter gene are used to permanently transform a suitable plant. The transgenic population so obtained is then mutagenized, usually by EMS, and screened for an altered expression of the reporter gene. This approach has been described as “targeted genetics.” Mutants isolated in this manner are altered in a specific response, e.g., a hormone-mediated expression of a gene, and can lead to isolation of other genes that regulate the expression of the first gene.

5. OTHER TECHNIQUES

5.1. Homology-Based Cloning

Several proteins involved in metabolic pathways or signal transduction serve common functions in

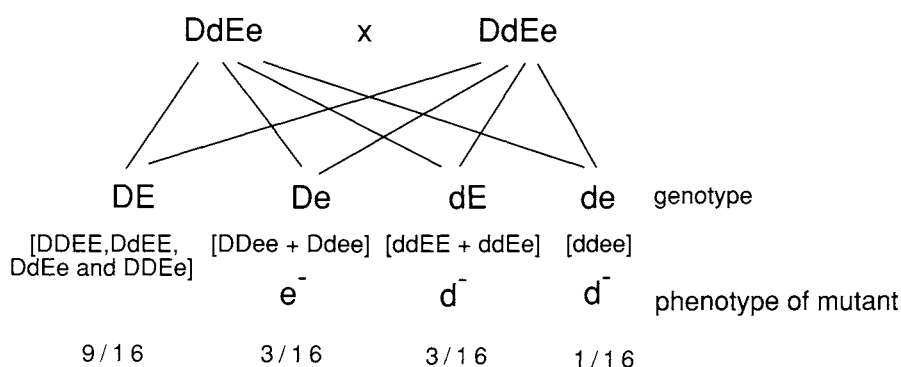


FIGURE A1-17 Model showing epistasis. Genes *A*, *B*, *C*, *D*, *E*, and so on encode proteins *A*, *B*, *C*, *D*, *E*, ..., which are assumed to regulate sequential steps in the elaboration of a developmental pattern, say the apical-basal pattern in embryogenesis. Mutants *d* and *e* are known and let us assume that they are recessive mutations, which confer distinct or opposite phenotypes. To determine whether gene *D* acts ahead of gene *E*, the progeny of a cross between two plants heterozygous for alleles *d* and *e* at both genes is analyzed statistically. If one mutant phenotype, say *d*⁻, is found in significantly larger numbers than *e*⁻, it is concluded that gene *D* is epistatic to gene *E*. In the figure shown, phenotype *d*⁻ outnumbers *e*⁻ by 1/16. Epistatic analysis works only if the two genes are unlinked and the mutant alleles produce distinct phenotypes; also, it is meaningful only for genes that regulate discrete steps in a related event or pathway. If two genes regulate the same step, then the phenotype may be intermediate in character or may reflect the dominant gene.

bacteria, yeast, animal, and plant systems. Because signal transduction research in bacteria, yeast, and animal systems is generally ahead of that in plant systems, it is usually possible to take conserved sequences of genes encoding these proteins and use them as probes to isolate clones from a plant cDNA library. (Alternately, the conserved sequences may be amplified by PCR to obtain related plant sequences directly.) This method, known as homology-based cloning, has proved fruitful in the isolation of many plant cDNAs and genes encoding components of signaling systems, such as protein kinases, protein phosphatases, small GTP-binding proteins, heterotrimeric G proteins, calmodulin, and Ca^{2+} /calmodulin-dependent protein kinases. The method, however, is of limited use in the isolation of genes that occur uniquely in plants or that have not been described in animal, yeast, or bacterial systems.

5.2. DNA Microarrays

The expressed genes are usually isolated by a differential screening approach in which a cDNA library is screened with labeled single-stranded cDNA, which, in turn, is obtained from mRNA extracted from tissue after $+/ -$ treatments. Variations on this theme include subtractive hybridization and differential display. In the past ten years, a new technique has evolved to screen a large number of genes simultaneously. The technique has evolved to screen a large number of genes simultaneously. The technique utilizes short short cDNA sequences or synthetic oligonucleotides of known sequence, and is ideal for organisms for which a large collection of expressed sequence tags (ESTs) exist (e.g., *Arabidopsis*). Full length cDNAs can also be used, if space permits. The cDNAs are applied by microsyringes and anchored to a solid support (e.g., a glass slide) in a high density array, usually by robotic arms. RNAs extracted from two different developmental stages (or experimental conditions) are converted to single-stranded cDNAs. The cDNAs are labeled with two different fluorescent tags for identification, mixed, and hybridized to the microarray in a competitive fashion. A high resolution scanner then quantifies the amount of fluorescent label in each cDNA spot, thus, allowing an assessment of which genes are being up- or downregulated. The genes still need to be isolated and cloned for functional analysis, and genes with low levels of expression are liable to be missed. Nonetheless, the technique provides a powerful tool for screening of hundreds of genes simultaneously and identifying the set of genes whose expression may be affected by a particular developmental stage or hormonal or environmental factor.

5.3. Yeast One- and Two-Hybrid Screens

As explained in Box 2-3 in Chapter 2, yeast cell lines with known mutations provide a very good tool for identification/confirmation of function of a protein encoded by a cDNA or gene sequence. They also are useful in the isolation of cDNAs coding proteins that interact with known sequences of DNA or for determining interactions between proteins. These assays, known as yeast one-hybrid and two-hybrid screens, are important techniques in determining signal transduction pathways and are briefly summarized here.

The yeast one-hybrid screen can be used to isolate a cDNA coding for a protein that binds to the response element for a hormone. Indeed, isolation of the first cDNA that coded for an auxin response factor 1 (ARF1), which binds to an auxin response element (AuxRE) in the promoter of auxin responsive genes, was accomplished in this manner. A mutant strain of yeast that lacked the enzyme for synthesis of an amino acid, histidine (*his3-1*) was used; mutant cells die unless supplied with histidine, which provided a selection strategy. The other selection strategy was to use β -galactosidase enzyme activity. Two constructs were introduced into the mutant cell line. Each construct had four tandem repeats of an AuxRE fused to a minimal promoter and was placed upstream of the coding sequence of the yeast histidine biosynthesis gene *HIS3* or the *lacZ* gene, which codes for β -galactosidase. The cell line was then transformed with a yeast vector containing fusions between random *Arabidopsis* cDNAs and the yeast *GAL4* transcriptional activator (Fig. A1-18A). The rationale was that a plant cDNA coding for a protein that binds to AuxRE will be able to induce *HIS3* and *lacZ* expression via the activation domain of the *GAL4* transcription activator. Yeast cells expressing the protein-*GAL4* fusion can be selected through their *HIS* autotrophy and β -galactosidase activity. Of the 1.2×10^8 cells screened, 500 *HIS* autotroph colonies were obtained, 5 of which also showed β -galactosidase activity. All 5 cDNAs encoded the same protein ARF1. Subsequent binding assays and *in vitro* gel mobility shift assays confirmed that ARF1 specifically binds AuxRE and is responsible for the auxin inducibility of gene expression.

The yeast two-hybrid system is used to identify interactions between two known proteins or to screen for partners that interact with a known protein. The selection works in the same way as in the one-hybrid system. In this case, however, the *HIS3* or *LacZ* marker genes are cloned behind a yeast promoter containing the yeast *GAL1* upstream activating sequence (UAS), and two different yeast vectors are used to cotransform the mutant strain (Fig. A1-18B). For one vector, the cDNA

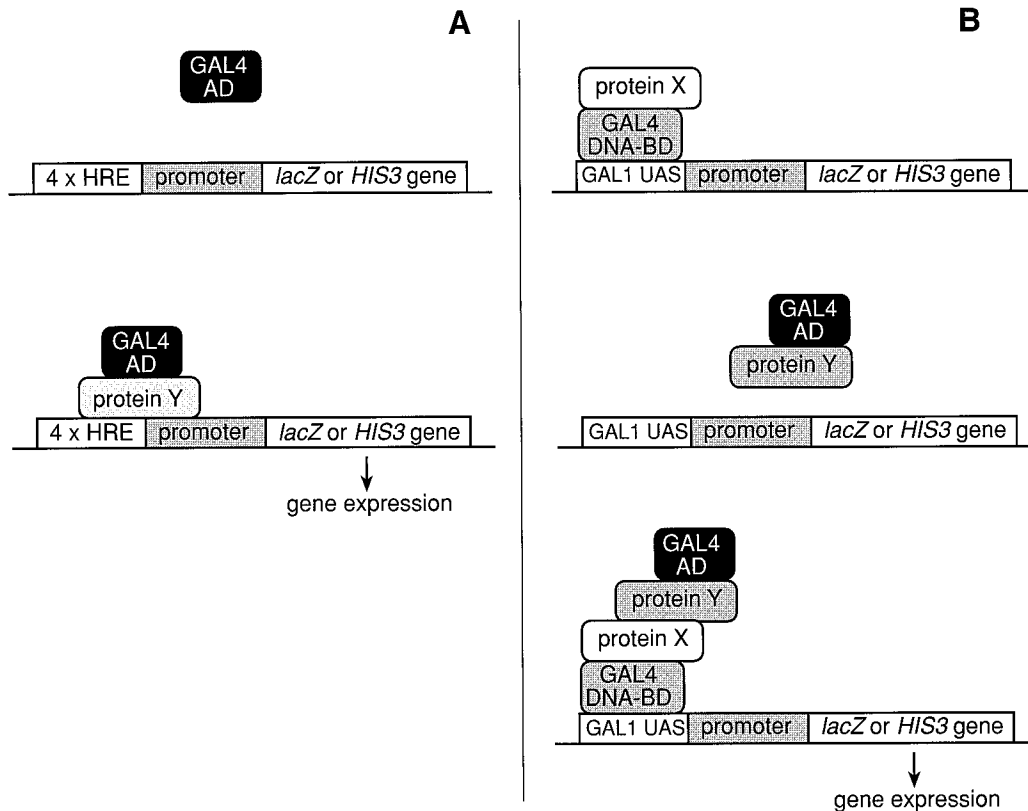


FIGURE A1-18 Genetic selection for plant signaling components in yeast. (A) The yeast one-hybrid system. The activator domain (AD) of GAL4 (a transcription factor from yeast) alone is not able to bind to the hormone response elements placed in tandem (4 × HRE) upstream of a minimal yeast promoter (top). Expression of the marker genes is only expected when the GAL4-AD is fused to a plant transcription factor containing the proper DNA-binding domain (protein Y) (bottom). (B) The yeast two-hybrid system is used to demonstrate interactions between known components in signal transduction or to identify proteins (Y) that interact with a known protein (protein X). Protein X is fused to the DNA-binding domain of GAL4 (GAL4 DNA-BD), which specifically recognizes the upstream activating sequence (UAS) from the GAL1 promoter (top). The GAL1 UAS is placed upstream of a minimal yeast promoter. Protein Y is fused to the activation domain of GAL4 (GAL4-AD) (middle). An interaction between protein X and protein Y brings the DNA-binding domain in proximity to the activator domain and allows expression of the marker genes to be initiated (bottom). From Offringa and Hooykaas (1999).

encoding the target protein is cloned behind a yeast promoter so that a translated fusion is obtained between the GAL4 DNA-binding domain and the target protein. For the second vector, the cDNA to be tested is translationally fused to the activator domain of GAL4 and cloned behind a yeast promoter. Cotransformation of the mutant line containing the UAS marker(s) with both vectors should only lead to HIS^+ and/or $LacZ^+$ yeast colonies when the GAL4-binding domain and the GAL4 activator domain are brought in close proximity through interaction between target and test protein. Using the two-hybrid system, it was shown that ARF proteins interact with members of the AuxIAA family of proteins coded by a family of primary auxin responsive genes. Further details

about ARF and AuxIAA proteins are provided in Chapter 22.

Artifacts can be introduced by endogenous interacting proteins in yeast. *In vivo* and *in vitro* tests are mandatory for the confirmation of transcription factors identified by these methods and/or confirmed by biochemical means.

6. NOMENCLATURE OF GENES, MUTANTS, AND PROTEINS

Since the use of mutants and isolation and characterization of wild-type genes has become a major

technique for studying plant developmental phenomena, it is necessary to standardize the designations for mutants, wild-type genes, encoded proteins, etc. Unfortunately, there is as yet no uniform system of nomenclature that applies to *all* plants.

Researchers using *Arabidopsis* generally follow a system recommended at the Third International *Arabidopsis* Meeting, East Lansing, MI, April 1987. According to these recommendations: (i) the genotypes are italicized or underlined; (ii) the wild-type genotype is capitalized; (iii) the genotype of a mutant allele is lowercase; (iv) alleles are specified by a dash followed by a number, and if no allele is specified, it is assumed to be number 1; (v) all gene symbols should be in three letters; (vi) phenotypes are designated by the gene symbol, which is not underlined but has the first letter capitalized; and (vii) the gene product, protein, is capitalized and not italicized.

Many researchers using dicot and some monocot plants, by and large, follow the system just described; others follow different systems, but there is little uniformity.

Many genes and proteins are specified as to the source plant; e.g., *At* for *Arabidopsis thaliana*, *Ms* for *Medicago sativa*; *Os* for *Oryza sativa*, etc. However, sometimes AT, MS, or OS is used and some use it as a prefix before and some as a suffix after the gene name.

In this book, for the most part, the *Arabidopsis* convention is followed. In some cases, because of previous usage or author preference, the wild genotype or its mutant alleles are written with less than or more than three letters. If genus and species are identified, they are abbreviated for binomial, italicized, and written as a prefix before the abbreviation for the gene or protein.

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II

STRUCTURE AND METABOLISM OF PLANT HORMONES

Plant hormones are small, simple molecules of diverse chemical composition: indole compounds, terpenes, adenine derivatives, steroids, aliphatic hydrocarbons, and derivatives of carotenoids or fatty acids. At least two are gases. In the context of rooted habit and iterative mode of growth, plant hormones are not produced in specialized glands, but in most parts of plants. Plant hormones are required in miniscule quantities and their concentrations at sites of activity are precisely regulated. Section II of this book deals with the structure and synthesis of plant hormones and their homeostatic regulation.

Plant hormones fall roughly into two groups based on their functions, not their chemical affinities. In one group are hormones that are involved in growth-promoting activities, such as cell division, cell elongation, pattern formation, tropic growth, flowering, and fruit and seed development. Hormones in the other group play important roles in plant responses to wounding and biotic and abiotic stresses. Among the chapters that follow, Chapter 5 deals with discovery, general features, and methods of analysis and quantitation of plant hormones. Subsequent chapters deal with structure and metabolism of auxins, gibberellins, cytokinins, and brassinosteroids in the first group, and with abscisic acid, ethylene, and jasmonates in the second group. Some compounds, such as polyamines, systemin, and salicylic acid, are generally not accepted as hormones. Polyamines have roles in growth-promoting activities and in retardation of senescence, but share a synthetic link with ethylene. Systemin and salicylic acid are involved in plant defense. Short accounts of polyamines and systemin are presented in chapters on ethylene and jasmonates, respectively.

Bacteria and fungi have coevolved with plants. Nearly every plant hormone is also produced by some microbial organism. Certain strains of *Agrobacterium* have established an elegant and unique association with plants. They literally take charge of a small section of the host plant and divert its resources to their own nutrition. The machinery they use for inserting their genes in host genome is being exploited by researchers in the genetic manipulation of plants. The microbial synthesis of plant hormones and genetic transformation are included in Appendix 2 at the end of this section.

General Features of Plant Hormones, Their Analysis, and Quantitation

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1. DISCOVERY OF AUXIN AND OTHER HORMONES

It is a common observation that aerial parts of plants bend toward a unidirectional source of light. This bending is caused by uneven growth on the two sides of the stem, with the side away from the light source growing faster than the side facing the light source. A series of ingenious experiments by

several scientists over almost 50 Years established that the bending was due to a natural substance, indole acetic acid (IAA), that was produced in the tip and diffused downward, causing uneven growth on the two sides (Fig. 5-1). Because it was a diffusible substance, it could be collected and, when reapplied to plant systems, could give a measurable response, thus creating the first bioassay for a plant hormone.

In the 1950s and 1960s, several other naturally occurring substances, gibberellins (GAs), cytokinins (CKs), abscisic acid (ABA), and ethylene, were shown to influence many plant responses (although some of them had been discovered earlier). Along with auxin, they are among the major plant hormones, sometimes referred to as the "classical five." Note that we use the expression "auxins," "gibberellins," and "cytokinins." This is because there are several naturally occurring or synthetic compounds that show auxin-, or gibberellin-, or cytokinin-like activity. In contrast, ABA and ethylene are single compounds, although some of their metabolites may also show activity. More recently, brassinosteroids and jasmonic acid have also come to be recognized as hormones, and several other substances, salicylic acid, polyamines, oligosaccharins, and a small peptide (systemin), have been shown to act as growth regulators or signaling molecules.

During World War II and the years following, many synthetic substances were also discovered and found to affect plant growth and development, thus adding to the list of plant growth regulators. This list continues to increase.

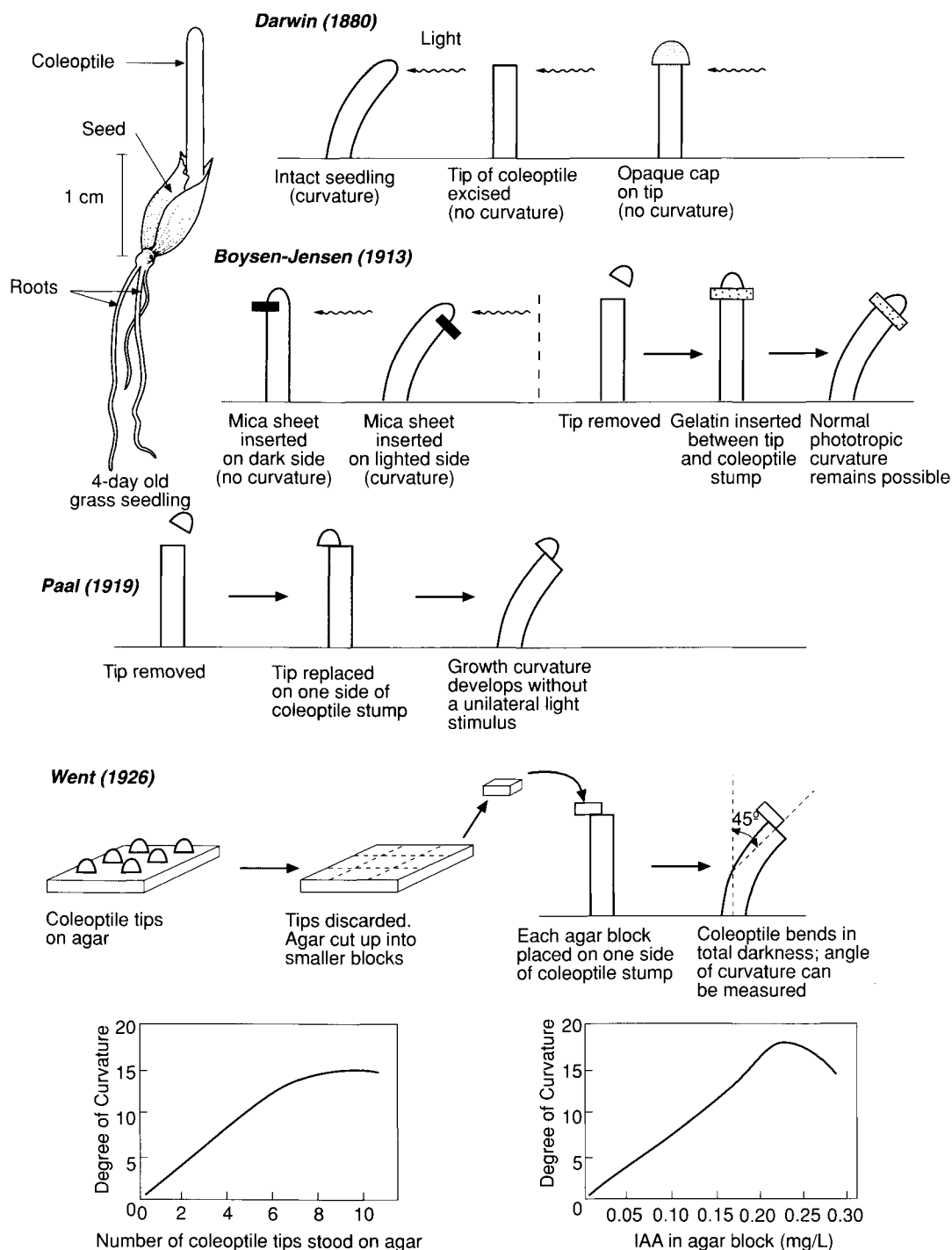


FIGURE 5-1 A summary of important experiments that established the existence of auxin in plants. Coleoptiles of grass seedlings were used in all experiments. Darwin (1880) illuminated the coleoptile of canary grass (*Phalaris canariensis*) from one side and noted that the coleoptile bent toward the light source. If the tip was cut off or capped, there was no response, indicating that light perception occurred in the tip. Since the bend occurred in the subterminal part, he correctly deduced that bending was due to the diffusion of some "influence" from the tip to the subterminal regions. Boysen Jensen (1913) placed thin mica or gelatin strips between the cut tip and the stump and noted that the "influence" was diffusible through gelatin, but not through the mica strip and thus established that the influence was chemical in nature. Paal (1919) showed that the coleoptile tip cut and placed on one side of the stump caused bending of the stump in the absence of unidirectional light. Went (1926) placed cut coleoptile tips on an agar plate and collected the diffusate; small blocks of agar with the diffusate were placed on cut stumps and elicited the

2. CHARACTERISTICS OF PLANT HORMONES

Each of the major classes of hormones brings about a variety of growth and morphogenetic responses, and each is **pleiotropic** in its effects. For example, auxins are involved in the regulation of cell division, cell growth, apical dominance, responses to directional stimuli (tropic response), and fruit setting, responses that are very different from each other. Not all responses are stimulatory. Auxins promote shoot growth, but at similar concentrations inhibit root growth. Second, several hormones may affect the same response; for instance, cell elongation is affected by auxins, gibberellins, and brassinosteroids; cell division is affected by auxins, cytokinins, and gibberellins. Thus, there is an **apparent redundancy** in control of the same response. Whether the same signaling pathway is involved and whether even the mechanics of the response are the same in each case is not known, but the phenomenon of redundancy is common. Third, plant hormones are **active at small concentrations**, usually in the nanomolar range, although some responses begin at even lower concentrations (10 to 100 picomolar, pM). Up to a certain concentration of the hormone, the response increases and then saturates (Fig. 5.2). For some hormones, higher concentrations may even become inhibitory (see Fig. 5.1).

Plant hormones are often reported to be active over 4 or 5, rather than 2 or 3, decades of concentration. These results from a response *in vivo*, or bioassays, do not necessarily reflect the concentration of the hormone at the target site because of problems in uptake, transport, and/or metabolism of the hormone in question (see Section 5 below). As more precise measurements of hormone concentration at the target site become possible, this conclusion may change. Fourth, transport of hormones from the site of synthesis to the site of action (target site) occurs in some cases, e.g., basipetal transport of auxin affecting lateral bud dormancy, but in others, the site of production and the site of action may be the same, e.g., ethylene production in fruit ripening. Moreover, **plant hormones are synthesized at several different sites** in the plant, not in any one specific gland or tissue. Thus, IAA is synthesized in young apical buds, young leaves, immature fruits, developing seeds, and so on.

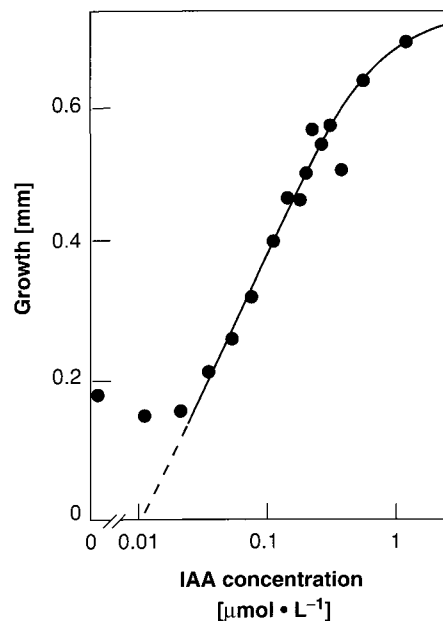


FIGURE 5-2 *Avena* coleoptile extension growth as a function of auxin concentration. Subapical segments (5 mm in length) from oat coleoptiles were depleted of endogenous auxin by floating in buffer solution (pH 4.7) containing sucrose ($20 \text{ g} \cdot \text{liter}^{-1}$) for 120 min, then different concentrations of IAA were added, and growth over the next 150 min was measured. Data are plotted as growth vs log IAA concentration. The minimum concentration of IAA that elicits a growth response varies from experiment to experiment, but usually ranges between 10 and 30 nM. The growth rate then increases proportionally to the logarithm of auxin concentration, reaching an optimal rate at about $1.0 \mu\text{M}$. Auxin concentrations in excess of $10 \mu\text{M}$ are superoptimal. Adapted from Cleland (1972).

3. HORMONE VS PLANT GROWTH REGULATOR

Because animal hormones¹ are usually produced in a specific gland or tissue, are transported to the target site, are usually more specific in their biological action, and are active in a more narrow range of concentrations, it has been suggested that the word “hormone” is inappropriate for plants and should be replaced with a term more suitable. Also, many synthetic

¹“Hormone,” as applied to animal systems, refers to a *naturally occurring-organic molecule that occurs in small concentrations, is produced in one site and transported to another site (target region), and brings about a specific biochemical or morphogenetic response.*

curvature response. This response could be measured, thus giving rise to the first bioassay. The diffusate was called “auxin” and was shown chemically to be indole-3-acetic acid (IAA). (Bottom) The oat (*Avena sativa*) coleoptile curvature response plotted against the number of coleoptile tips stood on agar (left) and against the concentration of IAA (right; note that supraoptimal concentrations of IAA can cause inhibition). Curiously, the first chemical determination of indole-3-acetic acid was made from human urine (Haagen-Smit, 1934), and only in 1941 was it proven unequivocally that IAA was also present in higher plants. Adapted from Taiz and Zeiger (1998).

compounds are equally, at times even more, active than the naturally occurring compound. For both these reasons, it has been suggested that it is better to call these substances plant growth regulators (PGRs) and include in that term both naturally occurring and synthetic compounds with growth-promoting activity.

However, that is putting too restrictive a definition on the term hormone. Plants have evolved differently from animals in several important ways, e.g., rooted habit, open form of growth, open differentiation, presence of cell walls, and autotrophic habit. It can be argued that having multiple sites of production or having the site of production and target site the same has selective advantage for plants that are subject to predation, even wanton damage, by a host of animals, including humans. Also, it is not necessary to assume that plant hormones *must* satisfy all criteria for hormones *sensu strictu* the animal world. The term hormone needs to be interpreted more loosely.

Furthermore, the term plant growth regulator or substance also has exceptions; many responses brought on by PGRs, e.g., stomatal closure, are not growth related. Accordingly, in this book, the term hormone is used in a broad sense to include those plant substances that occur naturally, in small quantities, and bring about a variety of specific responses. In contrast, the term PGR, is used to include hormones, as well as the various synthetic compounds with "hormonal" properties.

4. HORMONAL RESPONSES ARE SPECIFIC TO A PHYSIOLOGICAL STATE

It should be emphasized that plant hormone responses are tissue and time specific. Each response occurs at a certain time in plant development and is seen only in a specific tissue or organ. An example is gibberellin-induced elongation of lettuce (*Lactuca sativa*) hypocotyls. If lettuce seedlings are germinated and then transferred to light, the hypocotyls grow to about 3–4 mm in 72 h. However, if gibberellic acid (GA_3) is supplied to these light-grown lettuce seedlings, the hypocotyls elongate 400- to 500-fold over the control (Fig. 5-3). The response is shown by hypocotyls and petioles of cotyledons, not by roots. Moreover, if GA_3 is given in 4-h pulses, it becomes clear that hypocotyls are most sensitive to GA_3 application between 8 and 12 h after the start of experiment. Later applications of GA_3 , after 24 h, have very little effect.

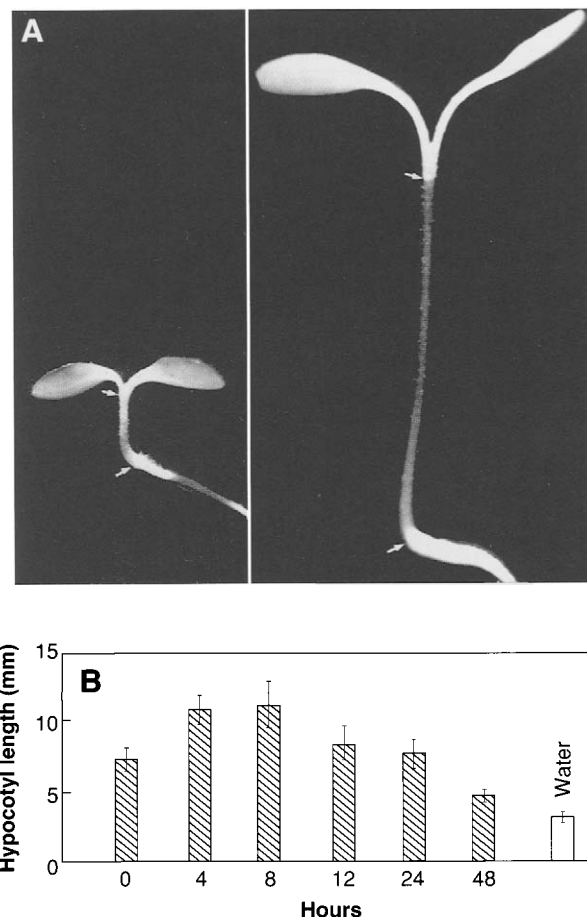


FIGURE 5-3 Effect of GA_3 on hypocotyl growth of light-grown lettuce (*Lactuca sativa*) seedlings. Seeds were germinated in the dark for 24 h, selected for uniformity of germination, brought to light, and given $10 \mu M GA_3$ or water alone (control). Growth was monitored over the next 72 h. (A) GA_3 -treated (right) and control (left) seedlings at the end of 72 h; arrows demarcate the hypocotyl. (B) Seedlings were given a pulse of 4 h GA_3 treatment at indicated times, and the length of hypocotyls was measured at the end of 72 h. For comparison, the hypocotyl length of seedlings grown in water for 72 h (water) is shown on the extreme right. Each bar in B represents the mean of 10 seedlings, and vertical lines represent 95% confidence limits. From Sawhney and Srivastava (1974).

The term "sensitivity" is used to denote the responsiveness of a tissue to a hormone. For a specific response, the sensitivity is maximal at a certain stage in development; it is less before and declines after that stage. The saturating concentration of a hormone for a particular response bears an inverse relationship to sensitivity of the tissue. If the sensitivity is declining or not at maximal, higher concentrations of the hormone may be needed to bring about the same magnitude of response.

Much of our information on hormone-mediated responses comes from bioassays or exogenous applica-

tions of hormones. The endogenous content of the hormone(s) in the response site is often not known, and very few studies on hormonal responses *in vivo* have tried to make this correlation. However, this situation is changing as better and more sensitive techniques of hormone detection and quantification have become available and are being used to reexamine some well-known physiological responses. Intriguing questions in this context are how does a response come to an end and what feedback mechanisms are operative? Beginnings are being made in elucidation of this phenomenon as well (see Chapter 19, Section II).

5. BIOASSAYS

A bioassay is the use of a plant, or plant organ or tissue, to measure its response to a specific plant growth regulator. Because there are many naturally occurring hormones and literally hundreds of their synthetic analogues and because these PGRs affect a wide spectrum of phenomena in plant growth and development, the number of bioassays is very large (see Yopp *et al.*, 1986).

5.1. Bioassays Serve Many Useful Functions

Bioassays are useful in many ways: (i) They are one of the major methods to determine the endogenous levels of a hormone in a plant organ or tissue with sensitivities of detection ranging from 1 to 1000 ng · g fw⁻¹. (ii) They are essential for monitoring the biological activity of a fraction during a hormone (or PGR) purification protocol. (iii) They are invaluable for testing new compounds—the range of responses elicited by the compound and its potency—in relation to natural hormones. (iv) They are useful for determining the relationship between the molecular structure of a compound and its biological activity, i.e., structure–activity relationships, and in devising new compounds.

The usefulness of a bioassay depends on its specificity and sensitivity, i.e., the response is given by only one class of PGRs and not others, and that a measurable response is obtained at relatively low concentrations of the PGR (e.g., < 10 ng · g fw⁻¹). It also depends on the precision with which the response can be measured and the ease with which the bioassay can be performed. For instance, the precision of measuring a flowering response is much less than that of measuring the elongation response, which in turn is much less than measuring the induction of an enzyme. The ease with which a bioassay can be performed includes such

factors as availability of a reasonably uniform plant source, ease of setting up the experiment in a laboratory, and costs of chemicals and apparatus.

5.2. Limitations of Bioassays

Bioassays suffer from three major limitations. (i) The actual concentration of the PGR at the site of action may be vastly different from that supplied because of conditions affecting the uptake of the PGR and/or its transport to the site of action. (ii) The PGR may be metabolized by living tissue, thus affecting its concentration; also, an inactive form of the PGR may be metabolized to an active form by the living tissues, or *vice versa*. (iii) Bioassay responses are subject to statistical error and usually are not accurate within 1 log of response. For these reasons, for qualitative and quantitative determination of specific hormones and PGRs, bioassays have been mostly replaced by newer, more precise analytical techniques (see Section 6). Bioassays remain indispensable, however, because they provide biological meaning to testing of new compounds and for determination of structure–activity relationships.

6. HORMONE EXTRACTION, ANALYSIS, AND QUANTITATION

6.1. Diffusible vs Extractable Amounts

A distinction is sometimes made between diffusible and extractable amounts of a hormone. The diffusible amounts are those that diffuse out from a cut surface and that can be collected in an agar block or some other receiver. They usually represent the fraction that is freely mobile. The first *Avena* coleoptile bioassay was performed using a diffusible hormone (see Fig. 5-1). In contrast, the extractable amounts represent those extracted by one or more solvents from a tissue homogenate. They include both the mobile fraction and one that may be bound to some other moiety or compartmentalized and, hence, immobile. The concept of diffusible hormone is not very useful because its relationship to endogenous content remains unknown. Henceforth, in this book, the term diffusible hormone is not used. The terms “free” and “bound” hormones are used, however. Bound hormone means that the hormone is chemically linked, or conjugated, to another moiety, often a sugar residue or an amino acid or a peptide. Conjugates may be hydrolyzed to release the free hormone (for IAA, see Chapter 6, Section 5.1.1).

6.2. Analytical Methodologies

6.2.1. Physicochemical Methods

Analytical methodologies for fractionation of a tissue homogenate and the subsequent identification and quantitation of hormone levels have undergone pro-

found changes since the 1980s. These methodologies utilize the high resolving power of high-pressure liquid chromatography (HPLC) and gas chromatography (GC), and identification of the molecular species by mass spectroscopy (MS).

BOX 5-1 HORMONE ANALYSIS: TOOLS OF THE TRADE

HIGH-PRESSURE LIQUID CHROMATOGRAPHY, LIKE other chromatographic methods, relies on the separation of different molecular species on the basis of their relative affinity to a stationary matrix, coated inside a column, and the carrier solvent that is used to elute them (Fig. 5-4). The higher the affinity of a molecule for the solid matrix, the greater the elution time (known as retention time). Eluates are monitored by a detector as they emerge out of the column and appear as peaks on the chromatogram. The height and size of the peak are measures of the relative amount of the eluate.

Gas chromatography also uses a column, a very long, narrow-bore, capillary column, which is coated on the inside by the matrix material, but in this case, the sample is volatilized when injected into the column. The volatiles so produced are eluted by a carrier gas, usually an inert gas such as helium. Their retention time in the column depends on both their affinity for the matrix material (the greater the affinity, the longer the retention time) and their volatility. Their emergence is monitored by a detector and plotted as peaks on a chromatogram. Most compounds are not easily volatilized. Hence, samples for GC are usually derivatized with methyl groups before injection into GC. GC has a much higher resolving capacity than HPLC, but because the samples are usually destroyed and cannot be recovered, it is used mostly for identification

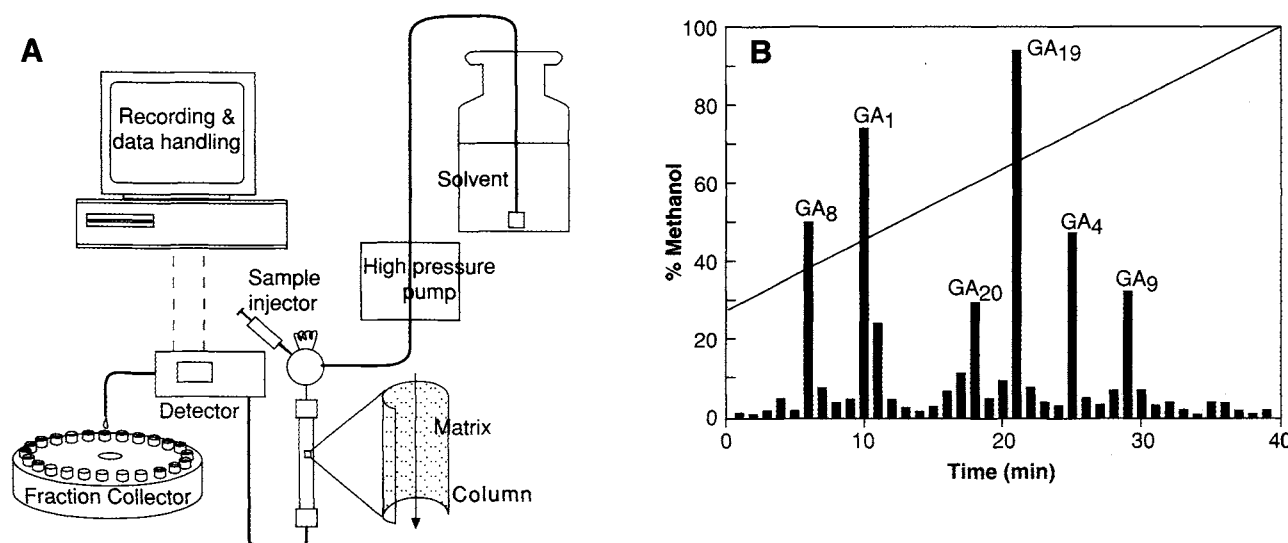


FIGURE 5-4 (A) Flow diagram of high-pressure liquid chromatography. The sample is injected into the column, coated on the inside by a stationary matrix (see cut through), and eluted by the solvent at a defined flow rate. Different molecular species in the sample pass through and exit the column in reverse order of their affinity for the matrix; the lesser the affinity, the shorter the retention time in the column. On exiting, they pass through a detector connected to a recording device, which plots the retention time as well as the quantities passing through. The samples are collected in a fraction collector for further analysis. (B) Separation of [³H]GAs by reverse-phase HPLC. The mixture of standards was injected onto an ODS Hypersil column (25 × 0.46 cm) and eluted at 1 ml/min over 40 min in a gradient of 28–100% methanol in water containing 50 μl/liter acetic acid. Radioactivity was determined by counting an aliquot of each 1-min fraction in a liquid scintillation counter. (Courtesy of Peter Hedden).

purposes (special columns have been designed for use in purification). Many different kinds of columns both HPLC and GC. Likewise, many kinds of detectors, suited for specific classes of compounds, are available on the market.

In a mass spectrometer, the sample is ionized and molecular ions (M^+) are separated according to their mass and displayed as mass peaks. Under certain modes of operation, the sample is bombarded with high-energy particles, such that the molecules are fragmented. These fragments are also charged ions, they can be collected on the basis of their mass and displayed as peaks. Since each molecule fragments in a specific way, it produces a characteristic array of ions called a mass spectrum, very much like a fingerprint (Fig. 5-5). Molecular ions, as well as fragment ions, provide an unambiguous identification of compounds.

Because unknown samples may have hundreds of different compounds, samples for MS are usually fractionated and purified by previous HPLC, or GC connected on-line to MS, such that only a small number of molecular species are present in any sample. Still, a full scan of the different ionic species is usually essential for knowing the range of compounds present in the unknown sample. Greater quantitative information can be obtained by a modification in which only a selected group of ions is monitored (selected ion monitoring or SIM). The combination of these techniques, fractionation of tissue samples by HPLC and identification by GC and GC-MS, provides as complete a picture as possible on the molecular species and metabolites of a hormone, and their respective amounts, in a sample.

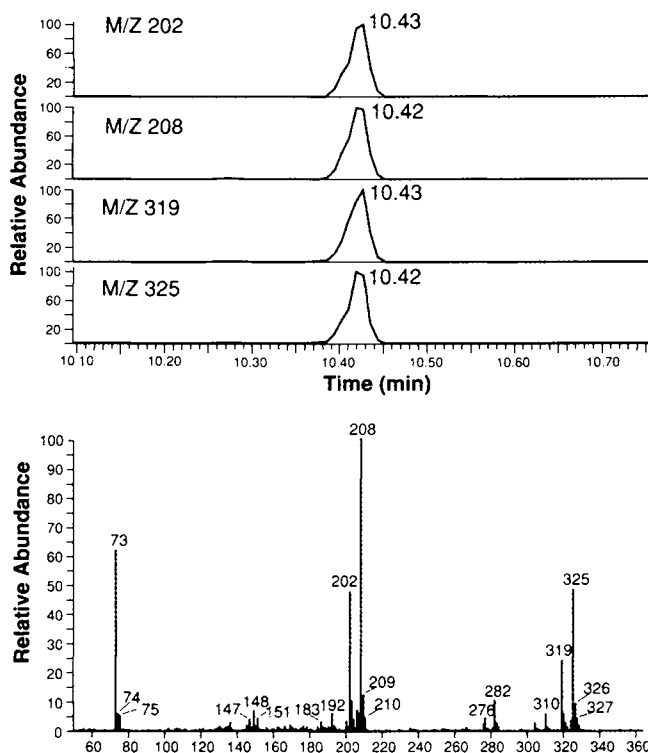


FIGURE 5-5 Full-scan mass spectrum (bottom) and mass chromatograms (top) for a mixture of IAA and [$^{13}\text{C}_6$]IAA as TMSi derivatives. The molecular ions are at m/z 319 and 325, respectively, and the fragment ions at m/z 208 and 202 are due to the loss of CO_2TMSi . The sample was analyzed using a Thermoquest GCQ mass spectrometer fitted with a fused silica WCOT BPX5 column ($25\text{ m} \times 0.22\text{ mm} \times 0.25\text{ }\mu\text{m}$ film thickness) (Scientific Glass Engineering). The sample was injected at an oven temperature of 60°C . After 1 min the splitter (50:1) was opened and the temperature was increased $20^\circ\text{C}/\text{min}$ to 200°C and then $4^\circ\text{C}/\text{min}$. The He flow was at a constant linear velocity of 40 cm/s . The injector, interface, and MS source temperatures were 220 , 270 , and 200°C , respectively. Courtesy of Peter Hedden.

Usually the tissues are frozen in liquid nitrogen, ground to a fine powder, homogenized, and extracted with one or more solvents to recover as much of the hormone and/or its metabolites as possible. The extract is partitioned against solvents (liquid-liquid or solid-liquid partitioning) to remove undesirable compounds, such as pigments, lipids, and phenolics, and the hormone extract is reduced to or dried and taken up in a small, defined volume. This crude extract is fractionated using HPLC. Fractions showing biological activity (by bioassays) may be further fractionated before being taken for identification on a GC or GC-MS. For ease of operation, precision and high resolution, and unambiguous identification of individual compounds, these methods far surpass the earlier thin-layer or open column chromatographic methods for fractionation and bioassays for identification and quantitation. The sensitivities and speed of these techniques are such that picogram quantities of hormones can be detected in a few milligram of plant tissues (Table 5-1), and the whole operation can be concluded within a day. To appreciate the elegance of these methods, it is fitting to remember that the isolation and identification of the first natural cytokinin, zeatin, required 60 kg of maize kernels and many liters of solvents (Letham *et al.*, 1964).

6.2.1.1. Internal Standards

The efficacy of these methods relies on the availability of chemically pure, but labeled, standards, which can be added to the plant tissue during extraction. These labeled standards have to be such that they are chemically identical, or nearly so, to the hormone being extracted so that it can be assumed that the labeled compound will behave similarly to the unlabeled endogenous compound throughout the isolation and analytical procedures. It is also very important that the labeled standards be prepared in such a way that they are stable under the extraction and analytical procedures. Two types of standards are used. Radioactively labeled standards, prepared by adding to or substituting ^3H or ^{14}C in a precursor, are useful in the extraction of hormones from plant tissues and their purification. Since radioactivity

can be detected in very small quantities, only a few milliliters of each fraction, from an HPLC column, need to be counted in a scintillation counter; 300–400 counts per minute (cpm) over a background count of 30–40 cpm provide adequate differentiation to determine which fractions should be pursued further. The ratio of radioactivity added before extraction to that recovered in a fraction reveals the recovery of the hormone and, thus, the efficiency of the extraction protocol. ^3H -labeled standards have a higher specific radioactivity than ^{14}C -labeled products and are preferred for most purification protocols.

Radioactively labeled compounds contaminate instruments and glassware and require a great deal of caution in their use. Accordingly, for most analytical work, pure standards labeled with heavy, but nonradioactive isotopes, such as ^2H , ^{15}N , and ^{13}C , are preferred and have proven invaluable for quantitative determinations. Their ions have a heavier mass than those of the natural hormone and can be separated in the MS. Usually, several heavy atoms are substituted in a molecule to improve the efficiency of mass separation. Stable, heavy isotope-labeled standards have been developed for most classes of plant hormones (e.g., [$^{13}\text{C}_6$]IAA, [$^2\text{H}_3$]ABA, [$^2\text{H}_2$]GAs), but their preparation requires great technical skill and hence they are still available only in selected laboratories. Ethylene does not require an internal standard because it can be identified and quantified using GC alone equipped with a flame ionization detector.

For quantitative work, internal standards are added in known amounts to the sample. Because the amount of labeled compound is known, determining the ratio of heavy isotope-labeled to unlabeled hormone reveals the amount of endogenous unlabeled compound, usually by use of a calibration curve (Croker *et al.*, 1994). These measurements are usually made in the SIM mode, with the MS set to monitor one or two ions of the endogenous compound and the equivalent mass-shifted ions of the internal standard.

6.2.2. Immunochemical Methods

Since the mid-1980s, immunological methods have also been established for the determination of several plant hormones. For immunological assays, an antibody must be prepared first. Because plant hormones are small molecules, unable to elicit an antigenic response in an animal (rabbit, mice, sheep), they are first conjugated to a protein [e.g., bovine serum albumin (BSA), ovalbumin, haemocyanin], injected into the animal, and antisera collected. As to which part of a hormone molecule is used for such attachment is an important consideration. For production of antibodies

TABLE 5-1 Free and Total IAA Contents in Single Oat Coleoptile Tips^a

Plant material	IAA content (pg · mgfw ⁻¹)	
	Free IAA	Total IAA (free plus conjugates)
Coleoptile tip	137 ± 27	399 ± 48

^aData are the mean ± SE ($n = 5$). From Ribinicki *et al.* (1998).

that recognize a specific part of the hormone molecule, it is important not to use that part of the hormone for attachment. Antibodies come from different cells in the spleen; hence, they are polyclonal antibodies (PABs) and show varying degrees of specificity toward different parts of the hormone–protein conjugate. These parts recognized by specific antibodies are referred to as “epitopes.” PABs may be partly purified to provide greater specificity toward one or another epitope. PABs, however, are unsuitable for large-scale quantitative work because that type of work requires an assured supply of well-characterized antibodies. To get this supply, it is essential to make monoclonal antibodies (MAbs). MAbs are produced by macerating the spleen and fusing the cells with tumor or myeloma cells. Spleen cells, by themselves, do not grow in the culture medium, but myeloma cells do. Thus, hybridization of spleen cells with myeloma cells produces a hybrid cell or a “hybridoma” that can grow in culture and also produce the antibody specific to the spleen cell. MAbs are purified by a combination of dilution and growth in culture, repeated several times, and their specificity to a specific epitope determined. They can be kept indefinitely in culture or frozen and stored for subsequent use.

6.2.2.1. Radioimmunoassay (RIA)

Radioimmunoassay requires a radioactively labeled hormone. A defined concentration of the radioactive hormone is incubated with a known quantity of antibody to which it binds, and then the free or unbound hormone is separated from that bound to the antibody by a suitable method (e.g., by dialysis or precipitation of the antibody by ammonium sulfate or by an anti-antibody followed by filtration or centrifugation). The radioactivity bound to the antibody is counted in a scintillation counter (Fig. 5-6A). This is the control (cpm 1). Another mixture includes the same quantity of antibody and the same concentration of the radioactive hormone, plus the plant extract with the unknown amount of hormone. The radioactivity bound to the antibody in this mixture is also counted (cpm 2). The more unlabeled hormone in the plant extract, the less radioactivity is bound to the antibodies. Thus, the difference in radioactivity between the control vs the sample is proportional to the amount of the unlabeled hormone in the sample.

The advantages of RIA are its relative simplicity and the high sensitivity provided by the use of radioactive compounds. However, there are several disadvantages as well: high specific activity-radiolabeled hormones and a scintillation counter are required, and they may not be easily available. Also, care is required in handling radioactive compounds.

6.2.2.2. Enzymeimmunoassays (EIA)

In enzymeimmunoassays, an enzyme replaces the radiolabel and the bound enzyme activity is measured, usually by a color reaction produced when a suitable substrate is presented (Fig. 5-6B). Monoclonal antibodies rather than polyclonal antisera are preferred because they are better characterized and can be produced in large quantities. The enzyme reactions are usually catalyzed by alkaline phosphatase or peroxidase. EIA requires that the enzyme be linked to the MAbs, which have been prepared against the hormone–antigen. The linkage can be accomplished in either of two ways: the enzyme can be covalently linked to the MAb (direct EIA) or it can be linked to a second antibody that specifically recognizes the MAb (indirect EIA). In direct EIA, the enzyme-coated MAb is incubated with a known quantity of hormone, plus/minus the plant extract. Subsequently, the substrate is presented, and the intensity of color is measured. The difference in color between the two assays provides a measure of the hormone content in the sample. In the indirect method, incubation mixtures consist of the same ingredients, plus the second antibody carrying the enzyme.

An EIA in which the antibody against the hormone–antigen is immobilized by adsorption onto a solid substrate is called an **enzyme-linked immunosorbent**

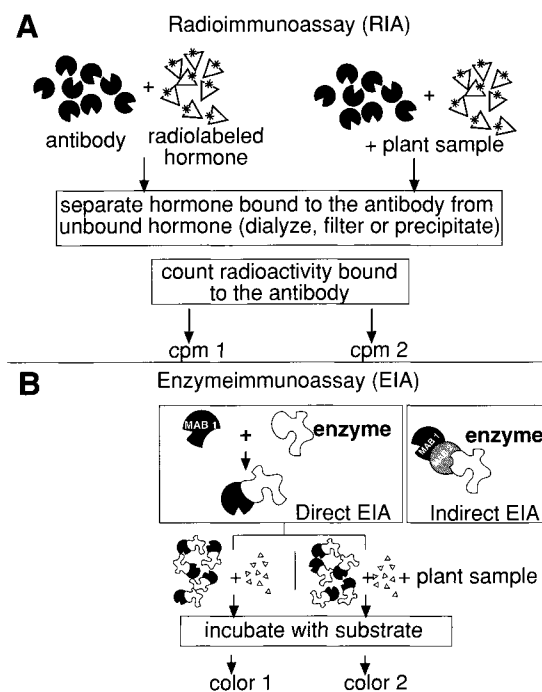


FIGURE 5-6 Radioimmunoassay (A) and enzymeimmunoassay (B) of hormones. For details, see text.

assay (ELISA). ELISAs have become the preferred method for quantitation of a hormone in plant material. Commercial assay kits are available for some hormones, such as ABA, IAA, and several cytokinins, which have microtiter plates with many small wells that are precoated with MAbs prepared against the hormone-antigen.

Both RIA and ELISAs are relatively easy to use and do not require costly equipment. Yet, the sensitivity of detection can be as low as 50 fmol, or about 10 pg of a hormone, such as ABA. In practice, however, these detection limits are often not reached because of impurities in the sample. Consequently, samples to be tested are purified as far as possible, preferably using HPLC, but purification leads to losses and therefore inaccuracies. For these reasons, results from immunological methods are usually verified by or calibrated against data obtained by GC and GC-MS.

6.2.2.3. Purification Protocols Using Immunological Methods

MAbs prepared against a specific antigen provide a very powerful tool for fast, reliable, and large-scale purification of the antigen or a close analog from an impure mixture. For such purification, MAbs are prepared as described earlier against the antigen of interest (if the antigen is a protein, it does not need to be conjugated to another protein, such as BSA). These MAbs are then covalently bound to a suitable solid matrix, usually a Sephadex bead. These beads come in standard sizes, and some are designed specially to provide linkage groups for binding to specific groups on MAbs. These special types of beads are referred to as affinity gels or simply Affi-Gels. It is important to ensure that the MAbs are bound to the Affi-Gel in a stable manner, a procedure that usually involves checking the stability of binding by the use of a radiolabeled antigen. The Affi-Gel bound to the MAbs can be packed in a column and then the impure mixture (or extract) containing the antigen is passed through the column. The antigen in the mixture adheres to the MAbs, while the rest of the mixture goes through. After a few washes to remove nonspecifically adsorbed proteins, the bound antigen can be eluted from the column by a suitable ionic buffer. In an alternate procedure, known as a batch method, the impure mixture may simply be mixed with a certain amount of MAbs bound to Affi-Gel, washed, and centrifuged and then the antigen is eluted from the MAbs as described earlier. Irrespective of whether a column or batch method is used, immunoaffinity purification provides a fast and easy method to purify large amounts of an antigen in one single step.

7. DETERMINATION OF HORMONE SYNTHETIC PATHWAYS

Three approaches are used to establish synthetic pathways for hormones.

7.1. Use of Labeled Precursors

The most common method is to feed labeled precursors to the plant or plant tissues *in vivo* or to a plant extract *in vitro* and follow the label in the products after varying periods of incubation. If the label is seen moving progressively from the precursor to A to B to C and so on, and finally the active hormone, one has a fairly good idea of the synthetic pathway.

For example,

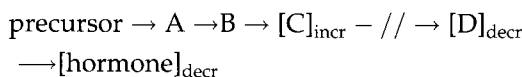
precursor \rightarrow A \rightarrow B \rightarrow C \rightarrow D \rightarrow active hormone

Initial studies are usually carried out using intact plants or plant parts *in vivo* and, later, extended to crude extracts from the same parts. Crude extracts, which are clear of cellular debris, i.e., **cell-free systems**, can contain all the enzymes and cosubstrates/cofactors necessary for biosynthesis *in vitro*. *In vitro* synthesis offers two advantages over *in vivo* work: (i) Before the addition of labeled precursors, the extract is usually dialyzed, which removes the endogenous hormone and thus prevents isotopic dilution. This is important because, as explained earlier, isotopically labeled hormones or analogs are added during an extraction protocol to facilitate the fractionation and identification of compounds of interest. (ii) It allows for eventual purification and characterization of the metabolizing enzymes, which can lead to cloning of their genes. Use of an *in vitro* synthesis system, coupled with the enormous analytical capabilities of fractionation by HPLC and the identification of compounds by GC and GC-MS, has proven invaluable for the elucidation of biosynthetic pathways for several hormones. These studies can be combined with the use of hormone synthesis mutants and synthesis inhibitors (see below), which can point out with great accuracy the precise steps in biosynthesis.

7.2. Synthesis Mutants

Each of the steps in biosynthesis is catalyzed by an enzyme. So another method relies on the use of mutants, natural or artificially created, that have a lesion in one of the enzymes in the biosynthetic pathway, and hence are deficient in the hormone. Of course, in order to recognize these mutants, they have

to show a phenotype that suggests a deficiency of the hormone. These are called **synthesis mutants**, and they have become a very powerful tool in determining the biosynthetic pathways of hormones. For example, a mutant that has a defective enzyme for the step C to D in the scheme given earlier would show a lack of, or deficiency in, the endogenous content of the hormone; it would also show an accumulation of products C and maybe others, before the blocked step as shown:



If a series of such mutants blocked in different steps is available, it is a wonderful aid in building up the biosynthetic pathway for the hormone in question. As explained in Appendix 1, the identification of mutant alleles allows cloning of the wild-type gene. Such cloning bypasses the requirement for purification of the enzyme protein.

7.3. Inhibitors of Hormone Synthesis

Still another method is to use inhibitors of biosynthesis, chemicals that block one or another step in the biosynthetic pathway. The result is the same as in the case of synthesis mutants, but because inhibitors often have side effects, which may be undesirable or may complicate results, the use of synthesis mutants, if available, is preferred.

Note: In addition to synthesis mutants, there are **response mutants**, which do not have a deficiency of the hormone, but where hormone signaling is affected such that the expected response to the hormone is not obtained. Similarly, there are chemicals that inhibit the action of a hormone, not its synthesis. Response mutants and **inhibitors of hormone action** are valuable tools in deciphering the mode of action of a hormone. They are referred to in many parts of the book, but mostly in Section IV.

8. REGULATION OF HORMONE LEVELS (HORMONAL HOMEOSTASIS)

Hormones are required for specific actions at specific times in growth and development, and it is important for the plant, not only to be able to synthesize the hormone, but also to inactivate it when not needed. Furthermore, hormones are required in small amounts, picomolar to micromolar quantities, and plants often produce far more bioactive hormone

than is actually required. Evidence comes from synthesis mutants that are leaky, i.e., the mutated allele is not a null allele, but is still able to produce a partly functional enzyme. Such leaky mutants often produce enough hormone to carry out many responses, although perhaps not all. Thus, the regulation of endogenous levels of bioactive hormones, or **hormone homeostasis**, is of prime importance to normal growth and development of plants.

Plants use three mechanisms to regulate endogenous levels of hormone: (i) regulation of the rate of hormone synthesis, (ii) inactivation of the hormone by conjugation with carbohydrates, amino acids, or peptides, and (iii) an irreversible breakdown of the hormone. Other means of regulating the levels of free hormone include transport to other parts of the plant and/or inactivation and storage in some compartment (Fig. 5-7).

Inactivation or breakdown of hormones and compartmentation in an inactive form are strategies that are regularly utilized. Similar inactivation or breakdown is also seen if plant tissues are presented with exogenous hormone in unnaturally large quantities or if the plant produces an excessive amount of the hormone as a result of a mutation or genetic transformation.

Before leaving this section, it is important to emphasize that mutants deficient in a particular hormone, or mutants or plants that have been transformed to overproduce a hormone, are invaluable tools in deciphering the physiological and/or biochemical roles of that hormone in plant growth and development. They point out with great specificity the particular roles a hormone plays and far surpass in accuracy the conclusions drawn from supplying the hormone to a whole plant or plant tissues and noting the effect(s).

Hormone homeostasis

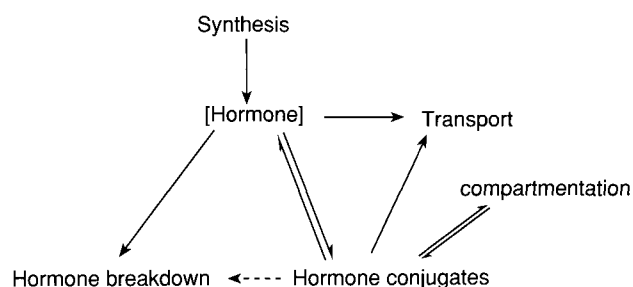


FIGURE 5-7 Summary diagram showing regulation of endogenous levels of a hormone.

9. CHAPTER SUMMARY

The discovery of indoleacetic acid as a natural hormone in plants in the 1930s was followed by the identification of several other natural hormones, gibberellins, cytokinins, abscisic acid, and ethylene in the 1950s and of brassinosteroids and jasmonates in the 1980s. In addition, many other natural and synthetic compounds are known that have roles in growth regulation or signaling. Plant hormones show several features that set them somewhat apart from hormones in animal systems. Each hormone generally brings about several responses that are distinct from each other, i.e., each hormone is pleiotropic in its effects. Moreover, several hormones may bring about the same response; thus, there is an apparent redundancy in their functions. Although plant hormones are active in small concentrations, they are usually active over several decades of concentration, which allows a certain flexibility in their use. Finally, plant hormones are not synthesized in a specific gland or tissue, but at many locations in the plant. These features of plant hormones are of importance to the survival of plants with their rooted habit. Hormones are required for specific actions in a highly time- and tissue-specific manner. The sensitivity of a tissue to a hormone is maximal at such times; it is less before, and declines after the stage is past. A bioassay measures a biological response to a hormone. Bioassays serve many useful purposes, but for quantitation of a hormone in biological samples, they have largely been replaced by physicochemical and immunochemical methods. The newer analytical methods, especially fractionation of samples by high-pressure liquid chromatography and identification and quantitation by gas chromatography, combined with mass spectrometry, allow unambiguous identification of hormones in plant tissues and their precise quantitation. Improvements in GC-MS techniques allow determination of hormone amounts in a single grain of cereal, or a single coleoptile tip. Immunochemical methods allow processing of large numbers of samples in a short time, as well as rapid methods of hormone purification. Synthetic pathways for hormones are determined by three methods; incubating plant tissues with labeled precursors and following the incorporated label in various products, use of mutants that are defective in one or another enzyme in the synthetic pathway, and use of inhibitors of biosynthesis. These methods, in combination with analysis and quantitation of products by GC-MS, have proven invaluable in defining the synthetic pathways for many hormones. Hormonal homeostasis is crucial to the orderly growth and development of plants. It is

maintained by a combination of several mechanisms, control over hormone synthesis, inactivation of bioactive hormone by conjugation, and hormonal breakdown.

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Auxins

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1. THE TERM "AUXIN" INCLUDES A VARIETY OF STRUCTURALLY UNRELATED COMPOUNDS

Since the original discovery of auxin as an indole compound that gave the grass coleoptile curvature (or growth) test (see Chapter 5, Fig. 5-1), the definition of auxins has been broadened to include not only indole-3-

acetic acid (IAA), but several other indole as well as nonindole compounds. Also, the functional characterization of auxin as a compound that gave the coleoptile curvature test has been broadened to include other functional tests, such as ability to cause rooting in stem cuttings and promote cell division in tissue/cell culture. Thus, the term auxin (from Greek "auxein", meaning "to increase" or "to grow") includes a spectrum of compounds that differ structurally and bring about a variety of auxin-type responses, albeit to varying degrees.

2. INDOLE ACETIC ACID (IAA) IS THE MAJOR NATURALLY OCCURRING AUXIN

Indole acetic acid is the most widely distributed, naturally occurring auxin in vascular plants, dicots, monocots, gymnosperms, and ferns. There are also reports of IAA being present in mosses and liverworts, as well as in some green algae (e.g., *Caulerpa*).

The chemical structure of IAA is shown in Fig. 6-1. IAA is a weak acid with a pK_a of about pH 4.85 and, in solutions at neutral pH, occurs in a dissociated state.

3. PHYSIOLOGICAL ROLES OF IAA

IAA is involved in nearly every aspect of plant growth and development, from embryo to adult reproductive plant. The processes regulated include pattern

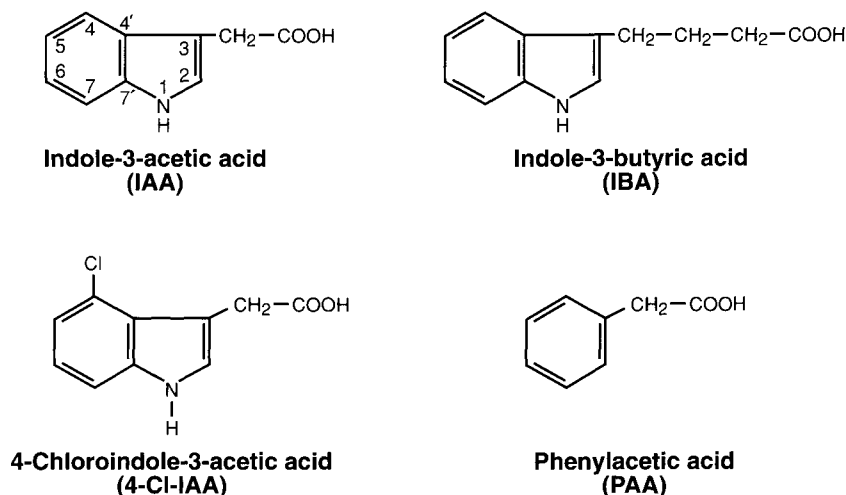


FIGURE 6-1 Chemical structure of indole-3-acetic acid and some other naturally occurring auxins. The numbering system for atoms in the indole ring is shown.

formation in embryo development, induction of cell division, stem and coleoptile elongation, apical dominance, induction of rooting, vascular tissue differentiation, fruit development, and tropic movements such as bending of shoots toward light or of roots toward gravity. Some of these responses are dealt with in Sections III and V of this book.

4. IAA BIOSYNTHESIS IN HIGHER PLANTS

Although IAA has been known since 1933, the biosynthetic pathway for IAA is still not completely understood. No natural or artificially created synthesis mutants have been identified, and inhibitors of IAA biosynthesis are just beginning to be identified. The approach to understanding the synthetic pathway(s), therefore, has been to feed a labeled presumed precursor(s) to plant tissues and see its incorporation into IAA. These measurements have been difficult because the products occur in low abundance and their radioactivity has to be measured against a background of radioactivity in precursors that occur in large abundance.

Until the early 1990s, tryptophan, an aromatic amino acid with structural resemblance to IAA, was thought to be the sole precursor for IAA. Since then, several novel modifications, including the use of stable isotopes, ²H and ¹⁵N, and tryptophan auxotrophs (i.e., mutants deficient in and requiring an exogenous supply of tryptophan to survive) have radically altered that view. It is now thought that IAA is synthesized by multiple pathways—that it can be derived from different precursors in different plants and,

indeed, in the same plant in different tissues or under different conditions and that its synthesis may be developmentally regulated.

Before discussing these results, let us see where the presumed precursors and IAA sit in the broader scheme of plant metabolism. The shikimic acid pathway is a very important metabolic pathway in plants (Fig. 6-2). Starting from erythrose phosphate and phosphoenolpyruvate, it leads to biosynthesis of the three aromatic amino acids—tyrosine, phenylalanine, and tryptophan—and also to synthesis of some very complex and important secondary plant products, including phenolics (e.g., anthocyanins, flavonoids, lignin) and alkaloids. It also leads to synthesis of IAA.

4.1. Tryptophan as a Precursor of IAA

Tryptophan (Trp), an amino acid with an indole ring (see Fig. 6-3), has long been regarded as the precursor of IAA in higher plants. Experiments utilizing radioactive tryptophan fed to intact or isolated plant tissues have been done for decades. The radiolabel incorporated into IAA is usually much less than expected, and there is always the possibility of microbial contamination (several microbes produce IAA via tryptophan, see Appendix 2); nonetheless, there is an overwhelming body of literature that indicates that IAA is synthesized from tryptophan. This conclusion has been confirmed in recent years using GC and GC-MS for bean (*Phaseolus vulgaris*) seedlings, carrot somatic embryos, and maize coleoptile tips. The pathway proposed in these experiments is via indole-3-pyruvic acid (IPA). It involves deamination of tryptophan to IPA and its subsequent decarboxylation to produce indole-3-acetaldehyde, which is oxidized to IAA

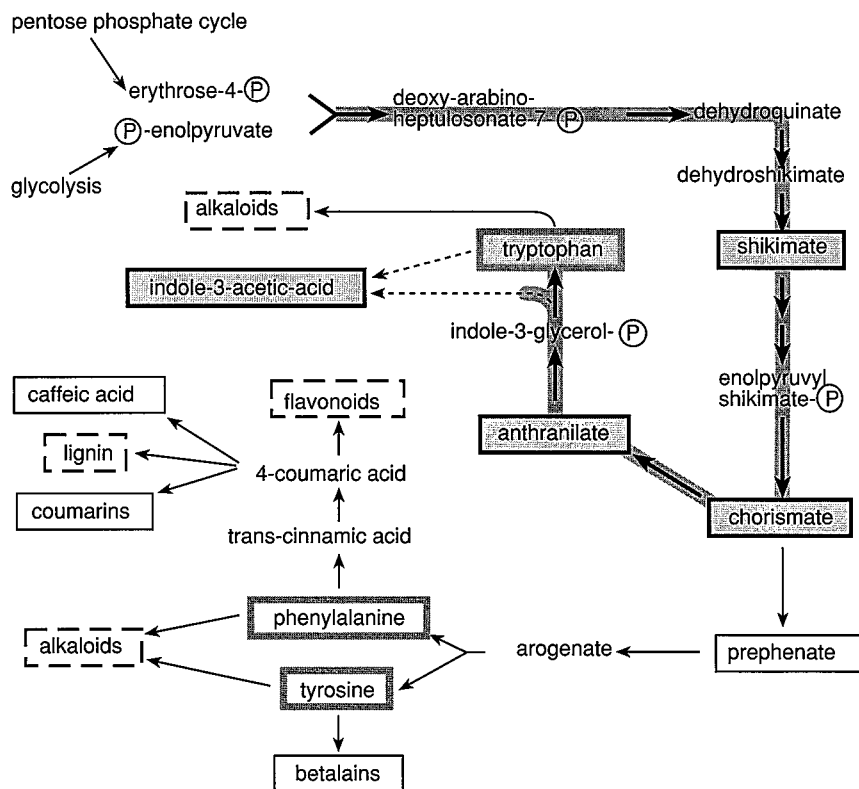


FIGURE 6-2 The shikimic acid pathway. This pathway occurs uniquely in plants and some microbes; it does not occur in animals. The route to tryptophan and indole-3-acetic acid and the major intermediates in that route are highlighted by thick arrows. Aromatic amino acids, tryptophan, phenylalanine, and tyrosine, and the important secondary plant products, lignin, flavonoids, and alkaloids, are enclosed in different boxes. Adapted from Mohr and Schopfer (1995).

(Fig. 6-3). This pathway is believed to be the common pathway in plants.

Another pathway *via* tryptamine has been recorded in a few instances, although the evidence is not very strong. This pathway also involves deamination and oxidative decarboxylation, but in reverse sequence, to produce indole-3-acetaldehyde.

The enzymes for these two pathways are present in homogenates, and a gene encoding a tryptophan decarboxylase has been cloned. However, an *in vitro* synthesis system is still being perfected (see Section 4.4) and the enzymes are not well characterized.

In the mustard family (Brassicaceae, to which *Arabidopsis* belongs), indole-3-acetonitrile (IAN) and its metabolites occur in abundance. It is considered possible, therefore, that IAA in this group of plants may arise from IAN (see Fig. 6-3). Evidence for such derivation in *Arabidopsis* is provided by mutants in nitrilase genes (e.g., *nit1*). Several genes encoding nitrilases (NIT1–NIT4) that hydrolyze IAN to IAA *in vitro* have been cloned. If the *NIT2* gene is expressed transgenically in tobacco, the transformed plants show increased sensi-

tivity to IAN and hydrolyze IAN to IAA at a faster rate than wild-type plants supplied IAN. Two other genes have been cloned in *Arabidopsis* that encode cytochrome P450 type hydroxylases. cDNA clones expressed in *Escherichia coli* were able to hydrolyze Trp to indole-3-acetaldoxime, the first step in the derivation of IAA from Trp *via* IAN. Thus, at least in *Arabidopsis*, and possibly other members of Brassicaceae and a few other families where nitrilase activity has been reported, IAA may indeed be derived from tryptophan *via* IAN.

Still another pathway for the derivation of IAA from tryptophan is seen in several microbes, such as *Pseudomonas* and *Agrobacterium*. The microbial pathway is covered in Appendix 2.

4.2. IAA Can Be Formed Independently of Tryptophan

There is also considerable evidence that IAA is formed independently of tryptophan. Evidence comes from two sources, both involving the use of stable isotopes: ^2H or ^{15}N .

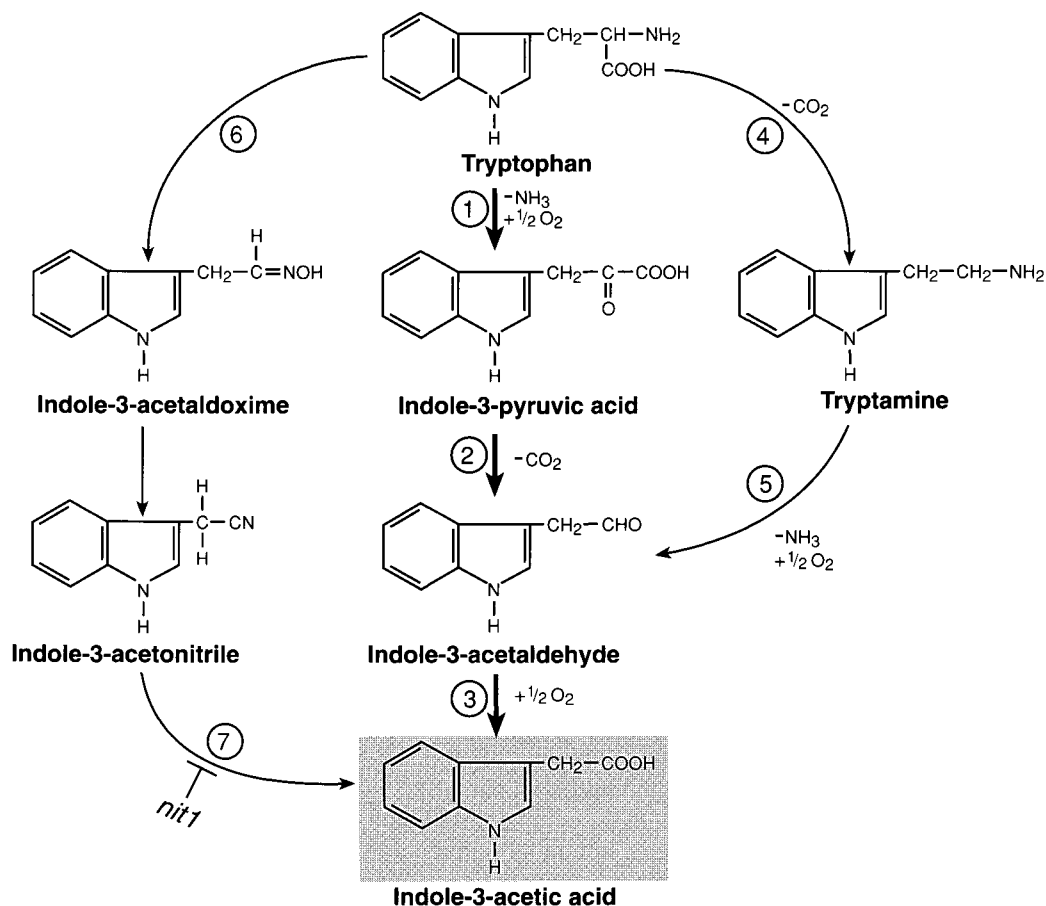


FIGURE 6-3 Tryptophan-dependent pathways of IAA biosynthesis. The pathway *via* indole-3-pyruvic acid is believed to be more common than the tryptamine or the acetonitrile pathway and is shown by heavy arrows. Enzymes involved are shown by numbers: 1, tryptophan transaminase; 2, IPA decarboxylase; 3, IAld dehydrogenase; 4, Trp decarboxylase; 5, amine oxidase; 6, cytochrome P450 hydroxylase; and 7, nitrilase, site of *nit1* mutation. Modified from Normanly *et al.* (1995).

4.2.1. Tryptophan Auxotrophs

Although synthesis mutants for IAA are not known, mutants in which tryptophan biosynthesis is impaired are known from maize and *Arabidopsis*. These mutants do not synthesize tryptophan and require exogenous Trp to survive; they are referred to as tryptophan auxotrophs. The biosynthetic pathway from chorismate *via* anthranilate to tryptophan is shown in Fig. 6-4.

The conversion of indole-3-glycerol phosphate to indole is catalyzed by Trp synthase α and of indole to tryptophan by two subunits of Trp synthase β , which together constitute the functional enzyme. The two subunits of Trp synthase β are encoded by two separate genes. A double recessive mutant was created in maize, in which both β subunits of the terminal enzyme were nonfunctional (the mutant had an orange pericarp and is called *orp*). The *orp* mutant does not synthesize tryptophan, but accumulates

almost 50 times as much total IAA (i.e., free IAA plus IAA conjugates, see Section 6.1) as the wild type. Incubation with [^{15}N]anthranilate efficiently labels both tryptophan and IAA in the wild-type plants, but only IAA in *orp* seedlings. Furthermore, *orp* or wild-type seedlings supplied with [^{15}N]tryptophan show no labeling in IAA.

The sites of lesion of three tryptophan auxotrophs, *trp1*, *trp2*, and *trp3*, in *Arabidopsis* are shown in Fig. 6-4. These mutants are conditional auxotrophs, i.e., plants grown at low light intensity are viable, but at high light intensity require an exogenous supply of Trp. The *trp1* mutant is defective in anthranilate phosphoribosyltransferase, *trp2* is defective in one of the subunits of tryptophan synthase β , and *trp3* is defective in tryptophan synthase α . Both *trp3* and *trp2* mutants do not synthesize Trp, as expected, but accumulate total IAA (IAA plus IAA conjugates). The *trp1* mutant accumulates anthranilate, does not synthesize Trp, and has

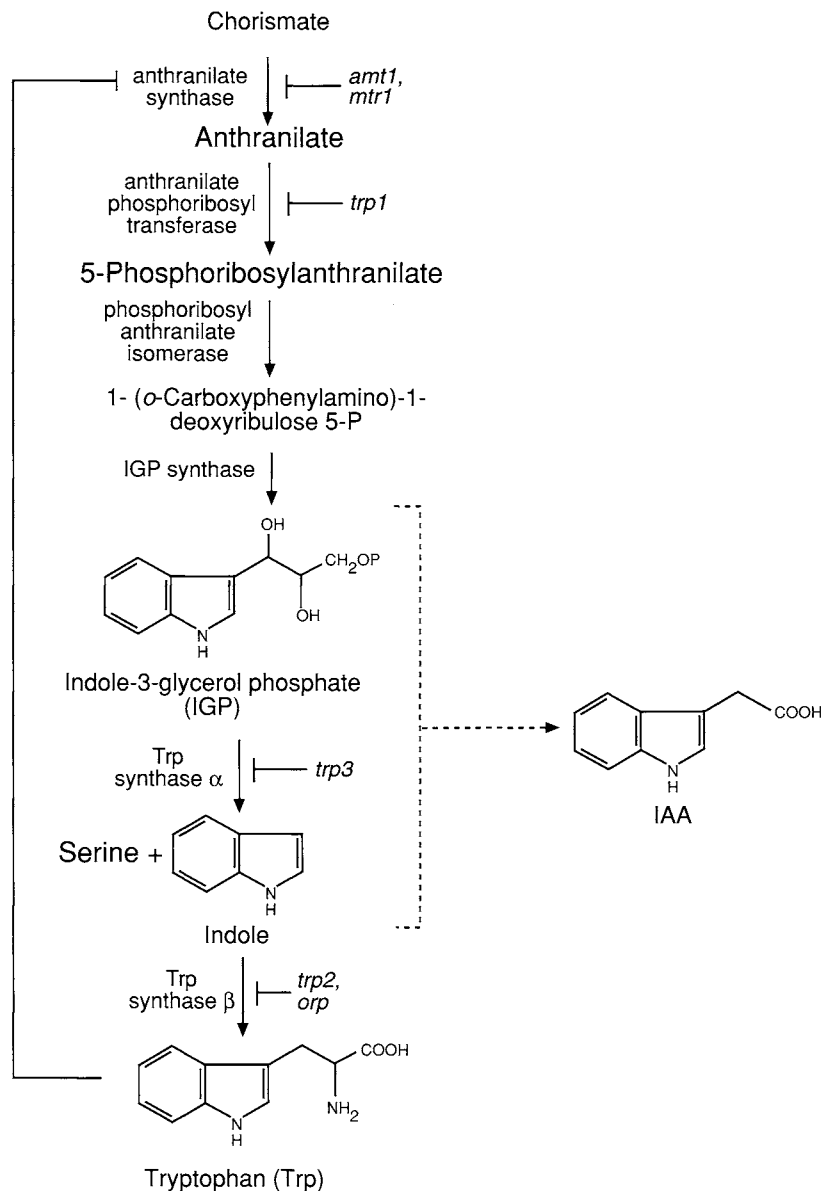


FIGURE 6-4 Possible tryptophan-independent pathway of IAA biosynthesis in plants. The Synthetic pathway for tryptophan from anthranilate is shown with enzymes catalyzing the various steps on the left and mutants on the right. (Left) The synthetic pathway for tryptophan. The sites for mutants are indicated. The mutants are *orp* in maize; *trp1*, *trp2*, *trp3*, and *amt1* in *Arabidopsis*; and *mtr1* (described originally as *MTR1*) in *Lemna gibba*. The possible origin of IAA from indole-3-glycerol phosphate and/or indole is shown by dashed lines; it may progress *via* indole acetonitrile or indole pyruvic acid. The figure also shows the feedback inhibition of anthranilate synthase by tryptophan. Modified from Normanly and Bartel (1999).

a phenotype suggestive of IAA deficiency, which confirms that the branch point for tryptophan and IAA lies after anthranilate.

4.2.2. Labeling of Endogenous Pools

If plants are grown in a medium enriched with heavy water ($^2\text{H}_2\text{O}$), deuterium is incorporated in a nonexchangeable manner into the benzene ring and

then into the indole ring. Using young plants of maize, *Arabidopsis*, pea, and carrot cell cultures, it has been shown that there is more deuterium labeling in the indole ring of IAA than in that of tryptophan. If tryptophan were the precursor of IAA, tryptophan should have at least the same if not more deuterium label than IAA. It is possible though that both compounds arise in a parallel manner from the precursors indole-

3-glycerol phosphate (IGP) or indole itself (see Fig. 6-4). In another experiment, ^{15}N -labeled tryptophan was fed to aquatic duckweed (*Lemna gibba*), and while more than 98% of the endogenous tryptophan pool was labeled, hardly any label was seen in IAA over a period of 5 days. This observation also casts doubt on tryptophan being the primary precursor of IAA.

Results from Trp auxotrophs in maize and *Arabidopsis*, as well as from stable isotope labeling experiments suggest strongly that IAA is derived from some precursor other than Trp, possibly indole-3-glycerol phosphate, or even indole. This conclusion agrees with results from bioassays using maize coleoptiles where anthranilic acid and other precursors give auxin-induced responses, but tryptophan does not. Interestingly, the *trp2* mutant not only accumulates indole, but also IAN, which suggests that IAN could also arise independently from Trp, possibly from indole.

4.3. *In Vitro* Synthesis of IAA

Bob Bandurski at Michigan State University, East Lansing, MI, and Jerry Cohen at the USDA/Agriculture Research Service, Beltsville, MD (now at University of Minnesota, St. Paul, MN), and their associates have contributed abundantly to studies on IAA synthesis and metabolism. In recent years they have also pioneered the development of *in vitro* synthesis systems for IAA, systems that have proven so useful for determining the synthetic pathways for gibberellins (see Chapter 7). Immature (or liquid) endosperm tissue from maize kernels and suspension cell cultures from the same tissue evidently have all the enzymes and cofactors required for IAA biosynthesis. The endosperm preparations can be frozen and, when thawed, retain the ability to synthesize labeled IAA from labeled precursors, such as anthranilate. Similar *in vitro* systems are also possible using seedling tissues. Using such systems, it has been possible to demonstrate that different tissues from maize plant utilize different pathways—the endosperm tissue produces IAA from Trp, whereas maize seedlings utilize mainly the Trp-independent pathway(s). These systems are still being perfected, but it is hoped that they will allow isolation and characterization of enzymes in IAA biosynthesis from tryptophan-dependent or -independent pathways and cloning of their genes.

The *in vitro* systems from maize are also useful in identifying compounds that inhibit IAA biosynthesis. Preliminary data already indicate that several indole compounds inhibit IAA synthesis from labeled tryptophan.

In summary, IAA biosynthesis in higher plants occurs in more ways than one and involves more than one precursor. There is substantial evidence for at least two tryptophan-dependent pathways and one or more tryptophan-independent pathways; the latter may utilize indole-3-glycerol phosphate, indole, or indole-3-acetonitrile as precursors. It also seems that Trp-dependent and -independent pathways may prevail in different plants or in the same plant at different times. As to which pathway is chosen seems to be controlled by developmental and/or environmental cues, which need to be elucidated.

4.4. Why Are There No IAA Synthesis Mutants?

Despite considerable effort, mutants defective in biosynthesis of IAA *per se* have not been obtained. The reasons are unclear, but there are at least two possibilities. Given the importance of IAA in regulating many processes vital to plant growth and development, it is possible that mutations in its synthesis are nonviable and, hence, are not detected in genetic screens. Second, if multiple pathways for IAA biosynthesis prevail in the same plant, genetic screens may not pick up an auxin synthesis mutant.

4.5. Sites of IAA Synthesis

Endogenous IAA levels are the highest in young tissues, shoot tips, young buds and leaves, young fruits, and immature seeds and are usually are much lower in older, mature tissues. Thus, it is reasonable to assume that young tissues are the sites of IAA synthesis. This supposition cannot yet be confirmed because the enzymes for IAA biosynthesis from tryptophan *via* indole pyruvic acid in higher plants are still not isolated, or their genes cloned. As mentioned earlier, genes for at least two steps in the tryptophan-derived IAN pathway have been cloned in *Arabidopsis*. *NITRILASE* genes are expressed in different tissues, root, leaf, stem, flower, and siliques to varying extents, but more studies are needed to relate their expression to young vs old tissues.

Within the cell, there seem to be two sites of IAA synthesis. Tryptophan is synthesized in the plastids, and, hence, the presumption is that IAA is also synthesized there, but there is also a cytoplasmic pool of tryptophan, which may contribute to IAA. Microbial production of IAA from tryptophan proceeds *via* indoleacetamide; in *Agrobacterium*-transformed tobacco cells, this intermediate is located in the cytoplasm. IAA conjugates (see Section 6) are also located in the cytoplasm.

5. REGULATION OF IAA LEVELS (IAA HOMEOSTASIS)

As mentioned in Chapter 5, levels of a hormone are precisely regulated in plant tissues by a variety of mechanisms, synthesis, conjugation, irreversible modifications, including catabolic breakdown, and transport and compartmentation (Fig. 6-5). Because the major enzymes involved in IAA biosynthesis in plants have not been isolated and characterized, we know little about the regulation of IAA biosynthesis by developmental or environmental cues. However, IAA can be reversibly converted to indolebutyric acid (IBA), another auxin (see Section 7). Also, regulation of IAA levels by conjugation or by catabolic breakdown is well known. Information on these topics has accumulated in recent years because many mutants have been discovered that overproduce or overaccumulate IAA. In the following, the chief mechanisms for conjugation and breakdown are discussed first, before a consideration of IAA-overproducing mutants.

5.1. IAA Conjugates

Most IAA in higher plants occurs in a conjugated form; the levels of free IAA (acidic form) are kept low. IAA can be conjugated to sugars or sugar alcohols *via*

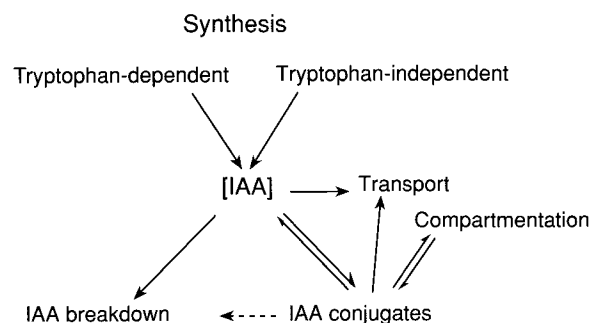


FIGURE 6-5 Summary diagram illustrating mechanisms for IAA homeostasis.

an ester linkage or to amino acids, peptides, or even proteins *via* an amide linkage (Fig. 6-6). The common sugars and sugar alcohols used for ester formation are D-glucose and, in maize, *myo*-inositol, although conjugates with arabinose and galactose are also known. The common amino acids used for amide formation are aspartic acid, glutamine, and alanine. Conjugates with glycine or valine are found in cultures that have been treated with auxin. IAA amides are the major conjugates in many dicots (e.g., *Arabidopsis*, legume seeds) and in pine shoots; in these cases, their concentration far exceeds that of IAA esters. In a study in *Arabidopsis*, IAA amides accounted for about 90% of

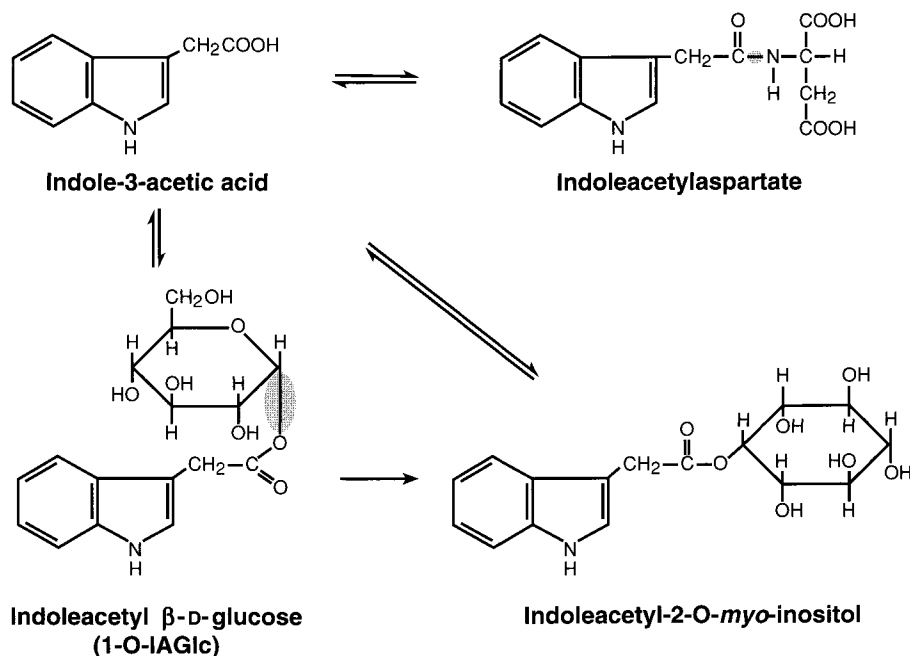


FIGURE 6-6 Reversible conjugation of IAA with sugars, sugar alcohols, and aspartic acid. Note that the carboxyl group of the acetate moiety is used for both linkages, but that in conjugation with amino acids, an amide, rather than an ester, link is formed.

the total conjugates, whereas IAA esters accounted for about 10%. Free IAA was about 1%. In other plants, such as maize, IAA esters are more common.

Conjugation renders the IAA biologically inactive, and conjugates are the predominant form in which IAA is stored in vegetative parts, as well as in developing, immature seeds. For instance, developing maize kernels were reported to accumulate IAA at the rate of $190 \text{ ng} \cdot \text{gfw}^{-1} \cdot \text{h}^{-1}$. Of the IAA synthesized, 97% was converted to the esterified form and less than 3% remained as the free acid. In the tryptophan auxotroph mutants of *Arabidopsis*, *trp2-1* and *trp3-1* (see Section 4.2.1., Fig. 6-4), lack of tryptophan synthesis was accompanied by a remarkable increase in the levels of IAA amides and, to a lesser extent, IAA esters, whereas the levels of free IAA were maintained nearly constant as in the wild-type plants (Table 6-1). Similarly, plants that have been genetically transformed by bacterial IAA synthesis genes to overproduce IAA (see Appendix 2) accumulate large quantities of IAA conjugates.

5.1.1. "Free" vs "Bound" IAA

Conjugated IAA is sometimes referred to as "bound" IAA (or "slow release" form of IAA) as opposed to "free" IAA, the acidic form. Several conjugates have been reported to give biological activity in bioassays, but the consensus is that the conjugates are inactive *per se*, that they are hydrolyzed by the plant

tissue, and it is the free IAA that gives the activity. Radiolabeling experiments indicate that the conjugated forms may be hydrolyzed to release free IAA during seedling growth or during plant tissue culture. For instance, in maize and bean, most of the free IAA in early seedling growth is believed to come from the hydrolysis of IAA conjugates.

5.1.2. Formation of IAA Conjugates and Their Hydrolysis

The formation of IAA esters with sugars and sugar alcohols has been studied in detail in maize. The first step in the formation of IAA glucosides is the conjugation of IAA to glucose *via* UDPG. This reaction is driven by a conversion of 1-O-IAGlc to 6-O-IAGlc, as well as the conversion of 1-O-IAGlc to IAA-*myo*-inositol. Other additions involving galactose, arabinose, and conversions may follow (Fig. 6-7). The manner in which IAA amides are formed is unclear.

Several enzymes in the formation or hydrolysis of conjugates are known and have been partially purified. The gene *IAA-Glucose synthase (IAGLU)* encoding the enzyme that catalyzes the first step in IAA glucosylation has been cloned from maize.

Enzymes catalyzing the hydrolysis of IAA conjugates with amino acids are being characterized using another approach, that of isolating *Arabidopsis* mutants defective in the hydrolysis of IAA conjugates with

TABLE 6-1 Levels of Free IAA, IAA Amides and IAA Esters in *trp2-1* and *trp3-1* Mutants and Wild-Type Plants of *Arabidopsis*

Genotype	Free IAA	IAA esters	Total IAA	IAA amides
Low light minus tryptophan				
Wild type	0.025	ND	0.06 ^b	
<i>trp2-1</i>	0.020	ND	2.11 ^b	
<i>trp3-1</i>	0.020	ND	0.43 ^b	
High light minus tryptophan				
Wild type	0.021	0.06 ^c		0.58
<i>trp2-1</i>	0.016	0.82 ^c		22.29
<i>trp3-1</i>	0.017	0.84 ^c		11.08
High light plus tryptophan				
Wild type	ND	0.05 ^c		0.86
<i>trp2-1</i>	ND	0.23 ^c		3.48

^a*Trp* mutants are conditional auxotrophs; they are unable to utilize tryptophan under high light conditions, but do so under low light conditions. Values given are $\mu\text{g} \cdot \text{gfw}^{-1}$. ND, not determined. Standard errors are not shown. Modified with permission from Normanly *et al.* (1993), © National Academy of Sciences, USA.

^bIncludes free IAA, IAA esters, and IAA amides.

^cAlso contains free IAA.

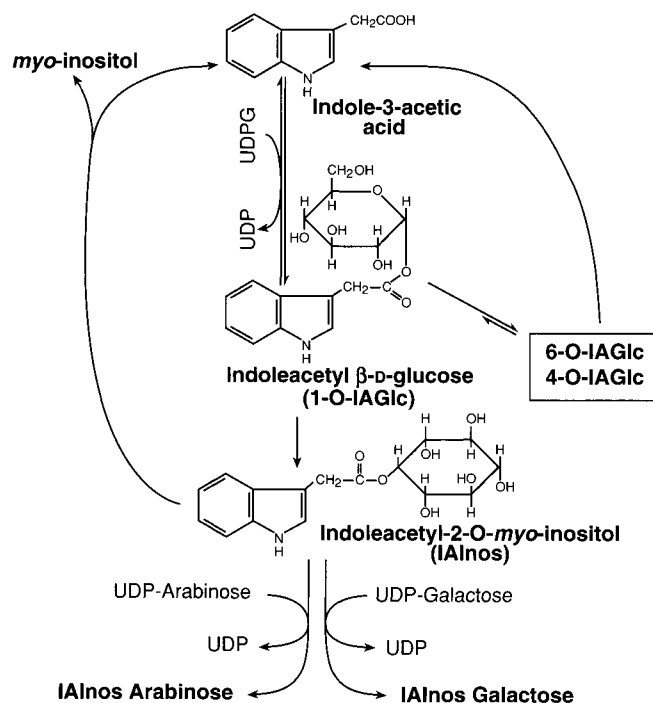


FIGURE 6-7 Synthesis of IAA esters in maize. UDP, uridine diphosphate; UDPG, uridine diphosphoglucose.

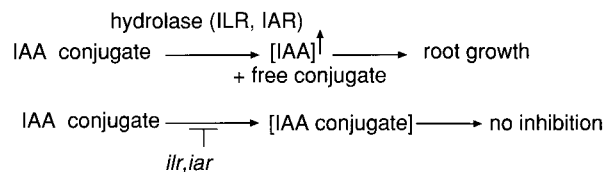
amino acids (for methodology, see Fig. 6-8). The wild-type genes for many of these mutants have been cloned and shown by expression in bacterial vectors to encode enzymes that have substrate preference for specific amino acid conjugates. For instance, ILR1, an amidohydrolase, specifically cleaves the amide linkage between IAA and leucine. ILR1 hydrolyzes IAA-Phe or IAA-Leu six to eight times faster than IAA-Ala, IAA-Gly, or IAA-Val. Many of these proteins reside in the endoplasmic reticulum (ER) (they carry a signal peptide for insertion into the ER and an ER retention signal), which suggests that at least some of the conjugate hydrolysis is carried on in the ER.

5.2. Breakdown or Irreversible Inactivation

Decarboxylation of the side chain and oxidation of the indole ring but without decarboxylation of the side chain are the two main pathways for irreversible inactivation of IAA. Both are oxidative reactions, but one proceeds with evolution of CO_2 , the other without.

5.2.1. Decarboxylation of IAA

Evidence for oxidative decarboxylation of IAA comes mostly from *in vitro* assays where IAA is supplied to cut tissues or segments. In these assays, several



Mutants defective in hydrolysing the amide bond are named as follows:

IAA-conjugate -*icr* (for IAA-conjugate-resistant)
 IAA-Leucine -*ilr* (for IAA-Leu-resistant)
 IAA-Alanine -*iar* (for IAA-Ala-resistant), etc.

FIGURE 6-8 Screen for isolation of mutants defective in hydrolysis of IAA conjugates. In a wild-type plant, an IAA conjugate yields free IAA by the action of a hydrolyzing enzyme. Free IAA inhibits root elongation growth (see Chapter 14). The screen for mutants utilizes this property of IAA. Mutagenized seedlings are grown in a medium with a specific amino acid conjugate in sufficient concentration to inhibit root growth. Seedlings that are not inhibited in root growth are potential mutants likely to be defective in the enzyme specific for that amino acid conjugate. Further rigorous screening and genetic characterization of the mutant follow.

degradation products of IAA, oxindole-3-methanol, 3-methyleneoxindole, 3-methyloxindole, indole-3-aldehyde, and indole-3-carboxylic acid, accumulate (Fig. 6-9). Peroxidases are ubiquitous in plant tissues, and many isozymes are known from horseradish, tomato, and tobacco, which show tissue-specific and/or environmentally induced activity. Peroxidases in the presence of hydrogen peroxide and cations such as Mn^{2+} degrade IAA to the decarboxylated products. However, whether IAA is degraded *in vivo* in this fashion is not certain. Although it is possible, the evidence for it being a major pathway in intact plants is not strong. The products of oxidative decarboxylation have been recorded only in a few plants (e.g., Scots pine, pea, lupin) and do not occur in significant amounts. Moreover, the gene for one of the peroxidase isozymes in tobacco was overexpressed under a constitutive promoter in transgenic tobacco, resulting in plants expressing 10 times as much peroxidase activity. The gene was also silenced in other transgenic plants by being introduced in an antisense orientation, causing a 90% decrease in peroxidase levels. The IAA levels, however, in the two treatments remained more or less the same.

Indole compounds are not only easily oxidized; they are also light sensitive. Thus, IAA solutions degrade in light, especially blue light, *in vitro*. The chemistry of photodestruction is not clear, but the products include indole-3-aldehyde and 3-methyleneoxindole. Because these compounds occur in plants, albeit in small amounts, it is possible that the photodestruction of IAA occurs *in vivo*. Riboflavin (a flavonoid) and violaxanthin (a carotenoid) pigments, which are present in large quantities in plants and which absorb in the

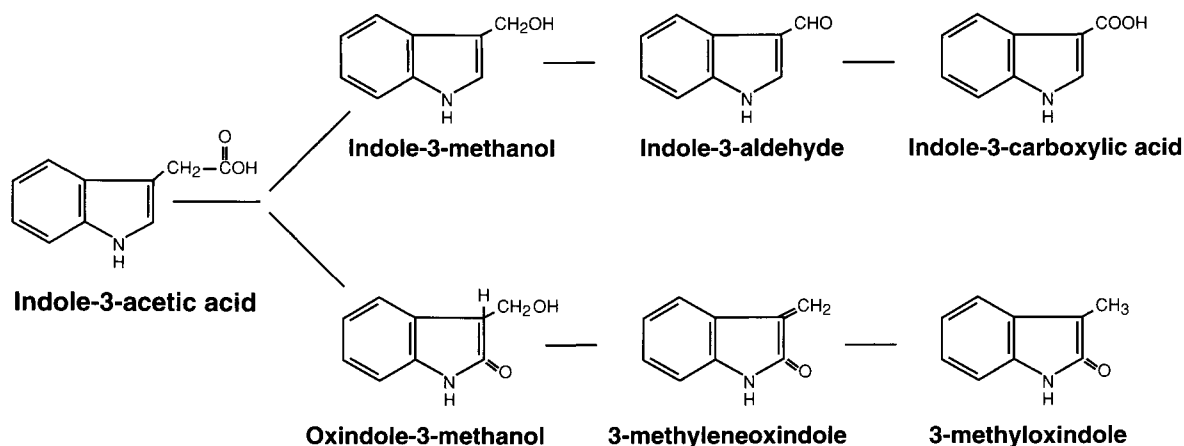


FIGURE 6-9 Some products of oxidative decarboxylation of IAA. Modified from Bandurski *et al.* (1995) with kind permission from Kluwer.

blue regions of the spectrum, could be the pigments involved in this photodestruction.

5.2.2. Nondecarboxylation Pathway

The nondecarboxylating pathway, in contrast, has been reported in many plants (e.g., rice, corn, bean, germinating seeds of *Brassica*, maturing seeds of *Ribes*; and among trees, poplar, spruce, and Scots pine) and is a regular feature of IAA homeostasis. In this pathway, the carboxyl group is left intact, but the indole ring is oxidized; the main products are oxindole derivatives, such as oxindole-3-acetic acid (OxIAA) and dioxindole-3-acetic acid (diOxIAA) (Fig. 6-10). In an alternative pathway, which seems to be more common, IAA is conjugated first to aspartate (IAA-Asp) and subsequently oxidized to oxindole-3-acetylaspargate (OxIAA-Asp). These products may be further modified in various ways by hydroxylations of the indole ring and additions of glucose residues to the indole ring. That this pathway occurs *in vivo* is supported by the observation that many of these products are labeled if plants are supplied with radiolabeled IAA. Also, the concentration of the oxindole derivatives formed endogenously often exceeds the levels of free IAA by severalfold.

5.3. Overproduction of IAA

Although synthesis mutants of IAA are unknown, there are many mutants that overproduce IAA. These mutants fall into two subgroups, one in which the wild-type levels of IAA are maintained by increased conjugation and/or breakdown of IAA. Mutants in this subgroup show the normal wild phenotype. In the other subgroup, the elevated levels of IAA cannot be controlled and cause phenotypic abnormalities. In this latter subgroup, some mutants show increased

conjugation, whereas in others, conjugation machinery seems to be defective.

5.3.1. Mutants Where IAA Homeostasis Is Maintained

We have already seen that in tryptophan auxotrophs, *trp3* and *trp2*, while tryptophan synthesis is impaired, far more IAA amides and IAA esters are accumulated than in the wild type (see Table 6-1). Although it is not proven, this increase probably results from increased synthesis of IAA, with free IAA levels kept low by increased conjugation.

Anthranilate synthase (ASA) is an allosteric enzyme that commits chorismate toward anthranilate and eventually tryptophan biosynthesis (see Fig. 6-4). Anthranilate synthase activity is also subject to negative feedback control by the end product, tryptophan. In mutants (*amt1*, *trp5*) of *Arabidopsis*, this feedback control is lost because of a defective subunit of ASA. As a result, much more tryptophan and IAA are formed than in the wild type. However, the free IAA levels are still kept low by the increased conjugation of IAA to inactive forms, and the plants exhibit a normal phenotype.

α -Methyltryptophan is a toxic analog of tryptophan. A mutant line of *Lemna gibba* (*MTR1*, the designation is changed in this text to lowercase *mtr1*), which is resistant to α -methyltryptophan, also shows a feedback-insensitive ASA, and thus maintains a high flux of tryptophan as well as of IAA. The levels of free IAA in this case, however, are not regulated by increased conjugation, but by increased breakdown, resulting in increased turnover of IAA.

5.3.2. Mutants with Disrupted IAA Homeostasis

In contrast to the mutants just described, there are other mutants of *Arabidopsis* and *Lemna gibba* where

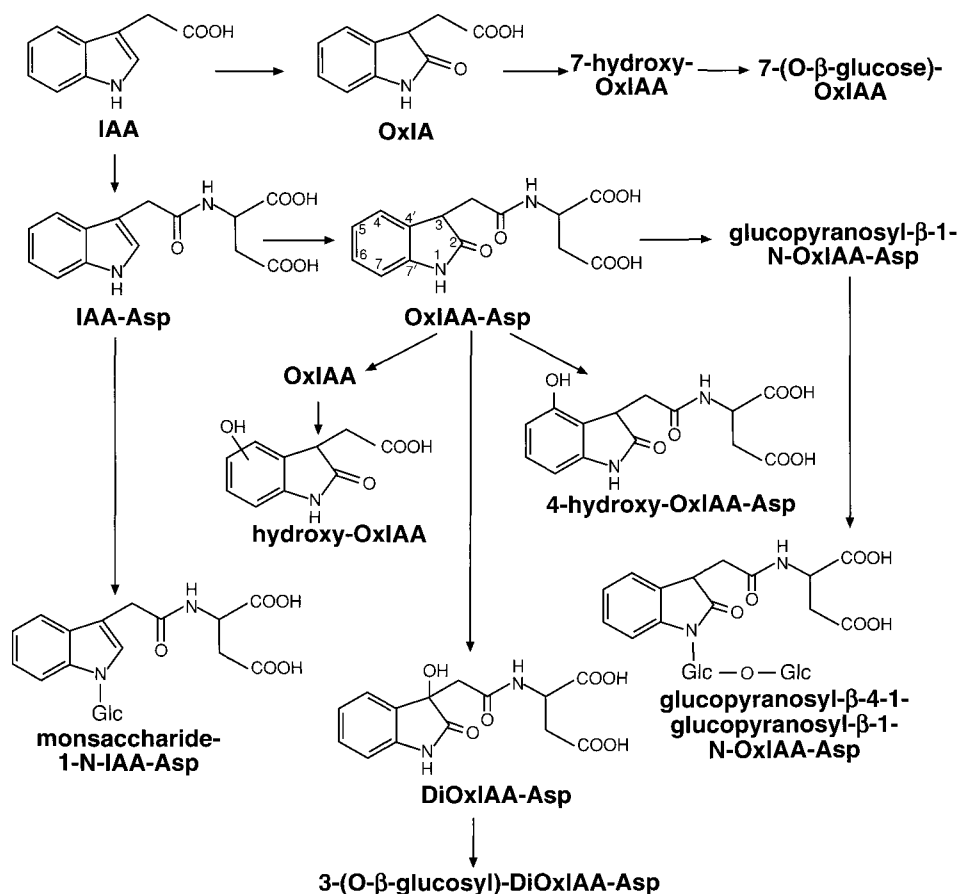


FIGURE 6-10 Nondecarboxylative oxidation of IAA. This pathway involves formation of oxindole derivatives, with or without prior conjugation with aspartate, and is the prevalent pathway in most plants. Modified from Normanly (1997).

IAA homeostasis is lost, resulting in phenotypic abnormalities. One mutant in *Arabidopsis*, described by different laboratories under various names (e.g., *hls3*, *sur1*, *rty*, *alf1*), shows two- to sixfold higher levels of IAA and IAA conjugates over the wild type (Table 6-2). It displays an abnormal phenotype: an increased number of lateral and adventitious roots, absence of the apical hook when grown in the dark, epinastic cotyledons and leaves, and sterility due to lack of flower development. These effects are obtained by the exogenous application of supraoptimal concentrations of synthetic auxins to wild-type plants. The *RTY* gene has been cloned and encodes a protein similar to tyrosine aminotransferases, which implies that an aminotransferase normally acts to limit IAA accumulation. How it does so, by inhibiting synthesis or enhancing conjugation, or other means, is not known.

A *sur2* mutant of *Arabidopsis*, at a different locus than *sur1*, has also been described. This mutant, while it shows some of the same abnormalities as the *sur1* mutant, shows elevated levels of IAA, but re-

duced levels of IAA conjugates, which suggests that the mutant is disrupted in IAA conjugation. Similarly, the *JsR1* mutant of *L. gibba* shows elevated levels of IAA but lacks conjugates and is believed to be disrupted in IAA conjugation.

In summary, IAA levels are precisely regulated in the plant by a combination of several mechanisms: regulation of biosynthesis of tryptophan, conjugation resulting in IAA esters and/or IAA amides, and catabolic breakdown, including oxidation of the indole ring. In some mutants in tryptophan biosynthesis, which results in IAA overproduction, free IAA levels are still maintained at normal levels by increased conjugation and/or a faster IAA turnover rate than in the wild type. In other mutants, the homeostasis is disturbed: both free IAA and conjugates are accumulated, or only IAA levels are increased while conjugate levels are low or lacking. This latter subgroup of mutants may well accumulate free IAA not because its synthesis is enhanced, but because IAA conjugation is disrupted.

TABLE 6-2 Mutants with Disrupted IAA Homeostasis

Mutant IAA overproducers	Plant	Phenotype	Reference
<i>JsR1</i>	<i>Lemna gibba</i>	Large frond size, dark green color	Slovin and Cohen (1988)
<i>rooty</i> (<i>rtv</i>), also known as <i>superroot1</i> (<i>sur1</i>), <i>aberrant lateral root formation1</i> (<i>alf1</i>), and <i>hookless</i> (<i>hls3</i>)	<i>Arabidopsis thaliana</i>	Abundant lateral and adventitious roots	King <i>et al.</i> (1995), Boerjan <i>et al.</i> (1995), Celenza <i>et al.</i> (1995), Lehman <i>et al.</i> (1996)
<i>superroot 2</i> (<i>sur2</i>)	<i>A. thaliana</i>	Similar to <i>sur1</i>	Delarue <i>et al.</i> (1998)

6. INHIBITORS OF IAA ACTION

Several synthetic compounds are known that inhibit IAA action, not its synthesis. These compounds are known as antiauxins [e.g., α -(*p*-chlorophenoxy) isobutyric acid or PCIB, see Fig. 6-11]. They have no auxin activity themselves, but specifically inhibit the action of auxin in a competitive manner; the inhibition is overcome by supplying excess IAA. The site of action is not known, although interaction with auxin receptor has been suggested.

Other synthetic substances, such as triiodobenzoic acid (TIBA) and naphthylphthalamic acid (NPA), are specific inhibitors of the basipetal, polar transport of IAA. They are considered in Chapter 13.

7. OTHER NATURALLY OCCURRING AUXINS

At least two other compounds with an indole ring occur naturally and give auxin-type responses in bioassays. A chlorinated derivative, 4-chloro-indole-3-acetic acid (see Fig. 6-1), occurs in members of the legume family, especially developing seeds, also pine seeds, and in bioassays can give up to 10 times the

activity of IAA. It is probably derived from indole-3-acetic acid by chlorination at carbon 4, and methylated forms as well as conjugates with aspartic acid are known. Indole-3-butyric acid (IBA) has been known as a synthetic auxin for a long time and is the major auxin used commercially for the induction of adventitious roots in stem or leaf cuttings (see Fig. 6-1; see also Chapter 14). In recent years, it has also been identified as a naturally occurring compound in several species (e.g., maize, pea, *Arabidopsis*). In both maize and *Arabidopsis*, IBA is derived from IAA via a chain elongation reaction similar to that found in fatty acid biosynthesis. IBA is also converted to IAA (Fig. 6-12). IBA and its conjugates are produced in mutants, such as *amt1* and *trp5* in *Arabidopsis*, which overaccumulate tryptophan and IAA. Thus, IBA may also be part of the machinery that maintains IAA homeostasis.

A nonindole compound, phenyl acetic acid (PAA, Fig. 6-1), was reported as a natural auxin. It has been reported in several crop plants, e.g., pea, tomato, tobacco, sunflower, barley, and corn; in these plants its concentration may be higher than that of IAA, although its biological activity is less than that of IAA.

8. SYNTHETIC AUXINS

The availability of a quantitative assay for auxin led to a widespread search for other compounds with similar biological activities. Much of this research done during the World War II was classified information and did not become generally available until after the war. Many synthetic auxins were discovered during this period.

The first compounds discovered were indole compounds, but with a side chain of propionic or

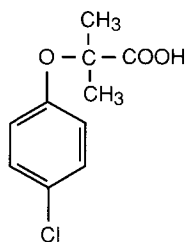


FIGURE 6-11 Structure of PCIB, an antiauxin.

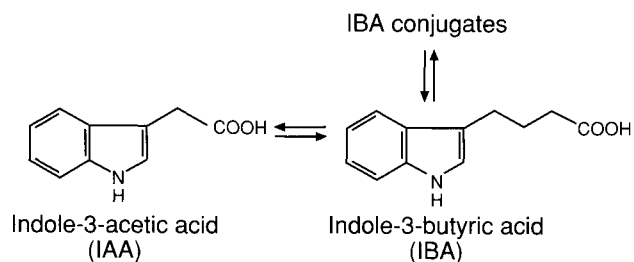


FIGURE 6-12 Interconversions of indole-3-acetic acid and indole-3-butyric acid.

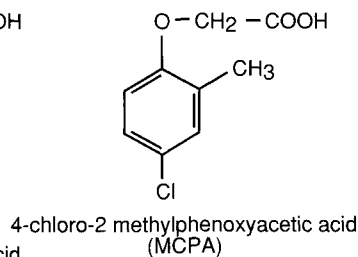
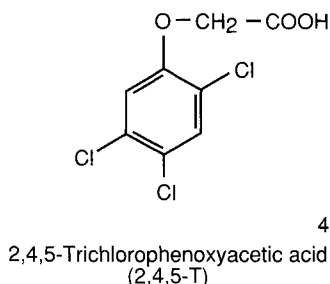
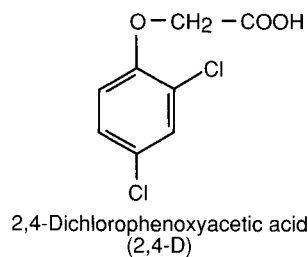
butyric acid, e.g., α -indole-3-propionic acid and α -indole-3-butyric acid. As we know now, indole-3-butyric acid occurs naturally in many plants, but for a long time it was known as a synthetic auxin only. Other compounds include chlorophenoxy acetic acids, such as dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), and MCPA; naphthalene acetic acid, e.g., 1-NAA (or α -NAA); and some benzoic acid derivatives with no side chain (Fig. 6-13).

Among the synthetic auxins, 1-NAA and 2,4-D are commonly used at relatively low concentrations to

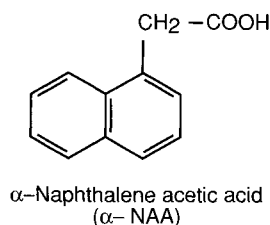
elicit auxin-type responses in cell growth, cell division, fruit set, rooting, and so on, although they affect these responses to varying degrees. For instance, 2,4-D is a much more effective inducer of cell proliferation in tissue or cell cultures than IAA. 1-NAA is often more active than IAA in the induction of rooting in stem cuttings. These compounds are relatively stable in plant tissues and are metabolized very slowly, if at all.

At high concentrations, 2,4-D, 2,4,5-T, MCPA, and the benzoic acid derivative dicamba act as selective herbicides (weed killers) or defoliants for broad-leaved plants or dicots. The reason for the herbicidal activity is not well understood, although excessive cell proliferation, epinastic bending, and other growth abnormalities are commonly observed. The herbicidal activity of 2,4-D or 2,4,5-T is much lower in grasses, probably because plants of grass family are able to metabolize these compounds by P450-type monooxygenases. Broad-leaved plants, in contrast, are generally not able to metabolize them, or metabolize them only to a small extent. In many cases we do not know how they get degraded.

Phenoxy-acetic acids



Naphthalene-ring-based



Benzoic-acid-based

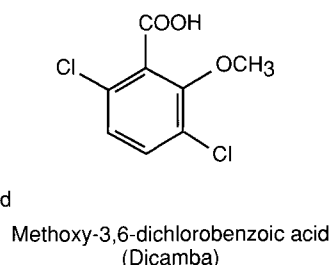
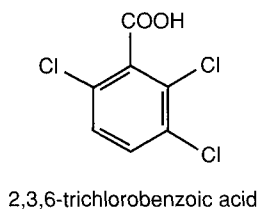


FIGURE 6-13 Some synthetic auxins. Phenoxy-acetic acid derivatives include 2,4-D, 2,4,5-T, and MCPA. α -Naphthalene acetic acid, also known as 1-NAA, is an active auxin, but 2-NAA is inactive. Benzoic acid derivatives, 2,3,6-TCBA, and Dicamba.

9. STRUCTURAL DIVERSITY OF AUXINS

How do synthetic auxins, such as 1-NAA, 2,4-D, and 2,3,6-trichlorobenzoic acid (or the natural auxin phenylacetic acid), with vastly different molecular structures from each other and from IAA, bring about "auxin-type" (IAA type?) responses is a question of fundamental importance for which we have no answer. We will refer to this question again in the introduction to Section IV of this book, but here it is relevant to point out that there is a possibility that synthetic auxins show IAA-like activity by inhibiting the deactivation of IAA or by promoting its synthesis. This may result in an elevation of free IAA levels beyond a threshold and lead to an IAA response. Some recent work provides support for this idea as well as negates it. For instance, 2,4-D was shown to inhibit IAA conjugation and to promote IAA synthesis from tryptophan in carrot cell cultures, but in other experiments, neither 2,4-D nor 1-NAA caused an increase in the free IAA levels.

10. CHAPTER SUMMARY

Indoleacetic acid is the predominant natural auxin in vascular plants and is responsible for regulating many aspects of plant growth and development. In higher plants, indoleacetic acid is synthesized by multiple pathways. In some, tryptophan serves as the precursor, whereas in others, the immediate precursors of tryptophan, indole-3-glycerol phosphate and indole, seem to give rise to IAA by parallel pathways. An advantage of having multiple pathways for synthesis of an important hormone is that plants are not likely to incur a deficiency of the hormone. The levels of free IAA in plant tissues are precisely regulated by a combination of several mechanisms. Conjugated esters and amides of IAA are common in plants. These conjugates are also believed to be the form in which IAA is stored in plants. In some plants, sugar conjugates stored in developing seeds have been shown to be mobilized during seedling growth. Irreversible breakdown of IAA also occurs. The more common pathway involves oxidation of the indole ring, with or without conjugation with aspartate, but without loss of the side chain; a minor pathway seen in some plants involves the decarboxylation of the side chain and may also involve oxidation of the indole ring. Although synthesis mutants of IAA have not been found, many mutants are known where large amounts of IAA are produced. In some of these mutants, the levels of

free IAA are kept to wild-type levels by conjugation and/or oxidative catabolism, whereas in others, IAA homeostasis is disturbed by an accumulation of large amounts of IAA with consequent changes in the growth pattern. Other naturally occurring auxins, besides IAA, include 4-chloro-indoleacetic acid, indolebutyric acid, and phenylacetic acid. Indolebutyric acid and IAA are known to be interconvertible, and IBA conjugates are known. Thus, in plants, IBA, in addition to serving as an auxin, may also serve to modulate free IAA levels. Many synthetic auxins, based on the ring structure of indole, naphthalene, or benzoic acid, are known and, if used in moderate doses, give auxin-type responses. In larger doses, many of them serve as herbicides to broad-leaf plants. The manner in which such structurally diverse auxins bring about plant responses is mystery.

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Gibberellins

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1. DISCOVERY

A disease of rice known as Bakanae or “foolish seedling” disease in which the plants grew inordinately long, became weak, and eventually fell over and died was known in Japan since the early 1800s (Fig. 7-1). The disease was attributed to infection by a fungus, *Gibberella fujikuroi*, an ascomycete, and Kurosawa in 1926 showed that the fungal extracts applied to healthy



FIGURE 7-1 Bakanae disease of rice. Rice plants infected with *Gibberella fujikuroi* are yellow and elongated relative to shorter and greener healthy plants. Courtesy of Dr. Yutaka Murakami.

plants produced the same symptoms as in Bakanae disease. In the 1930s, Yabuta and Hayashi purified the active compound from the fungal extract and called it gibberellin. This major discovery went unnoticed in Western countries until after the end of World War II. In the 1950s, researchers in England and United States perfected their own methodologies to isolate the active compound from *Gibberella* cultures and named the compound gibberellic acid. Because gibberellic acid application could induce elongation growth in genetic dwarfs of pea and maize, it was surmised that gibberellic acid must also occur in higher plants, a surmise proven correct by isolation of the first gibberellin from higher plants by MacMillan and Suter in 1957. Discovery of a gibberellin in higher plants led

to a flurry of activity not only in analysis of more and more plant extracts for identification and purification of other gibberellins, but also into discovery of various biological responses, other than stem elongation, elicited by GAs. As more GAs became known from fungi as well as higher plants, a system of nomenclature was established by MacMillan and Takahashi (1968). The first gibberellin extracted from higher plants was named gibberellin A₁ or GA₁; the so-called gibberellic acid, under the new nomenclature, became gibberellin A₃ or GA₃ [for very interesting accounts of these early years of gibberellin research, see Phinney (1983) and MacMillan (1996)].

2. STRUCTURE OF GIBBERELLINS (GAs) AND OCCURRENCE IN PLANTS

Gibberellins are defined by their structure rather than their biological activity. They are all cyclic diterpenes with an *ent*-gibberellane ring structure. Two main types of GAs are recognized, those with the full complement of 20 carbon atoms, the C₂₀-GAs, and the C₁₉-GAs, which have lost one C atom and possess a lactone (Fig. 7-2). Biologically active GAs in higher plants are C₁₉ compounds.

GAs occur widely in the plant kingdom—in angiosperms, gymnosperms, ferns, and lower vascular plants, such as *Psilotum* and *Lycopodium*. Among fungi, besides *Gibberella*, they are known from *Sphaceloma manihoticola*, another ascomycete, which causes the

“superelongation disease” of manihot (Cassava), and from *Phaeosphaeria* sp. L487. Gibberellin-like compounds have been reported in mosses and algae, although these reports need confirmation using modern methodologies.

The number of GAs is phenomenal. As of a recent count, 125 GAs are known and the numbers are still increasing. They are designated as GA₁ ··· 125, following the nomenclature of MacMillan and Takahashi (1968); the numbering in recent years has been in order of discovery. In addition to their being either C₁₉- or C₂₀-GAs, these GAs differ one from the other in number and position of -OH groups, -COOH groups, and -CH₃ groups and in the number and position of double bonds (Fig. 7-3). Although the number of naturally occurring GAs is large, the number of GAs that are biologically active is quite small (e.g., GA₁, GA₃, GA₄, GA₇, and a few others). Other GAs are intermediates in the biosynthetic pathway or are inactivated forms. Numerous GA derivatives and GA conjugates are also known, some only as synthetic compounds in the laboratory.

GAs are weak acids with a pK_a of ~ 4.8 and occur in a dissociated state at neutral pH. They have both polar and nonpolar groups and show a range of polarities. Factors that affect polarity include the number of OH and COOH groups, α- vs β-OH forms; and saturated vs unsaturated analogs, e.g., GA₁ vs GA₃. Substituents, such as methyl groups, and/or presence of sugar residues in ester linkage with a -COOH group or ether linkage with a -OH group further change the polarities.

Of the numerous GAs, most occur exclusively in higher plants, some are found in fungi, and some are common to both. In fungi, GAs are products of secondary metabolism; their function in fungal metabolism, if any, is not known.

3. PHYSIOLOGICAL ROLES OF GAs IN HIGHER PLANTS

GAs are involved in several important biochemical and morphogenetic responses. One very common response is the promotion of elongation in axial organs, such as stems, petioles, and flower pedicels. The response is seen most graphically in some genetic dwarfs of pea, or maize, and in “bolting” of rosette plants, such as cabbage, celery, and spinach. For example, seedlings of dwarf pea (*Pisum sativum*, genotype *lele*) have a dwarf habit and very short internodes if grown in light. If GAs are applied exogenously, the internodes elongate and the stems appear similar to

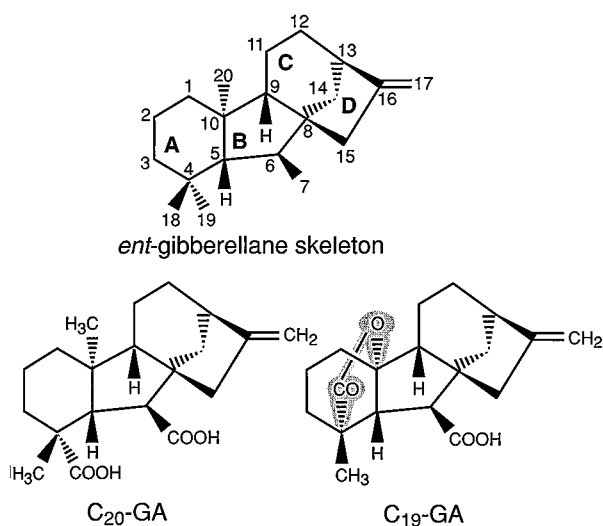


FIGURE 7-2 *ent*-Gibberellane skeleton and structures of C₂₀- and C₁₉-GAs. Four rings, A–D, and a carbon numbering system are shown for *ent*-gibberallane.

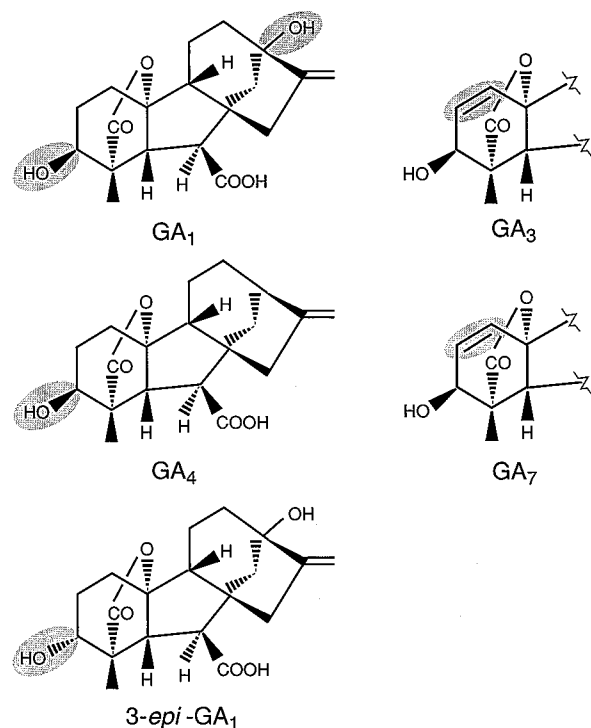


FIGURE 7-3 Structures of some important C₁₉-GAs. GA₁ and GA₃ carry hydroxyl groups both at C-3 and at C-13, but GA₃ has a double bond between C-1 and C-2; GA₄ and GA₇, a similar pair, have a hydroxyl group only at C-3. For GA₃ and GA₇, only part of the molecule (ring A) is shown. A distinction is also made between epimers. For instance, the OH group at C-2 or C-3 may be in the α or β position. The top four GAs have the hydroxyl group in the β position on C-3—they are β -hydroxylated GAs. Their epimers, such as 3-*epi*-GA₁ or 3-*epi*-GA₄, have much less biological activity than the β -hydroxylated forms. Note that C₁₉ or C₂₀ means a compound with 19 or 20 carbon atoms; C-19 or C-7 means carbon 19 or carbon 7 in the compound; GA₁₉ means gibberellin₁₉.

those in the normal or wild-type pea (*LeLe*) (Fig. 7-4). Rosette plants have short internodes and the leaves stay close to the ground. Flowering is associated with a rapid extension growth of the flowering spike, a phenomenon known as “bolting.” This growth can be brought on by a change in photoperiod or by an exogenous application of GA. The elongation response of GAs is covered in Chapter 15.

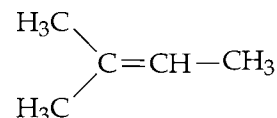
Seeds of many plants store large quantities of reserve foods—starch, proteins, and lipids—which are hydrolyzed following seed germination to provide energy and carbon skeletons as building blocks for growth of the young seedling. In cereal grains, e.g., wheat, barley, and rice, GAs induce the *de novo* synthesis and/or activation of several different enzymes for hydrolysis of storage products. This is one of the best studied plant hormone responses and has provided excellent material for studies into the molecular basis of gibberellin action (see Chapters 19 and 24).

Other responses include cambial reactivation in trees in spring, phloem tissue differentiation, germination of certain seeds, floral development, and, at low concentrations (~1000-fold less than required for shoot), stimulation of root growth. Gibberellin-like substances and GA derivatives are known to induce antheridia formation in fern gametophytes. Some of these responses are covered in Section III of this book.

4. TERPENOID PATHWAY IS THE SOURCE FOR MANY IMPORTANT COMPOUNDS

Terpenes, or terpenoids, are a large class of plant secondary products with a major role in defense against plant-feeding insects and herbivores (Gershenzon and Croteau, 1991). However, not all terpenoids act as secondary products. Many have important roles in primary processes, such as photosynthesis, stability of cell membranes, signaling, and as source compounds for several plant hormones.

Terpenes are characterized by their basic structural element, the five-carbon **isoprene unit**:



All terpenes are formed by condensation of two or more isoprene units and are classified on the basis of the number of isoprene units they contain (see Fig. 7-5). Ten-carbon terpenes containing two C₅ units are **monoterpenes**, 15-C terpenes are **sesquiterpenes** (three C₅ units), and 20-C terpenes are **diterpenes** (four C₅ units). **Triterpenes** and **tetraterpenes** have six or eight C₅ units, respectively. **Polyterpenes** have 10 or more C₅ units.

Isopentenyl diphosphate (IPP) is the five-carbon activated building block of terpenes, which isomerizes to dimethylallyl diphosphate (DMAPP). DMAPP is used for the synthesis of cytokinins (see Chapter 8). DMAPP and IPP are also the starting points for a series of head-to-tail condensations and cyclization to yield geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP). FPP is important in cell signaling, and its dimerization leads to the synthesis of triterpenes, including sterols (important components of membranes), and the class of plant hormones known as brassinosteroids (BRs). GGPP is the branch point for the synthesis of linear diterpenes, such as phytol for chlorophyll, or cyclic diterpenes, *ent*-kaurene, and ultimately all GAs. Dimerization of GGPP yields tetraterpenes, such as

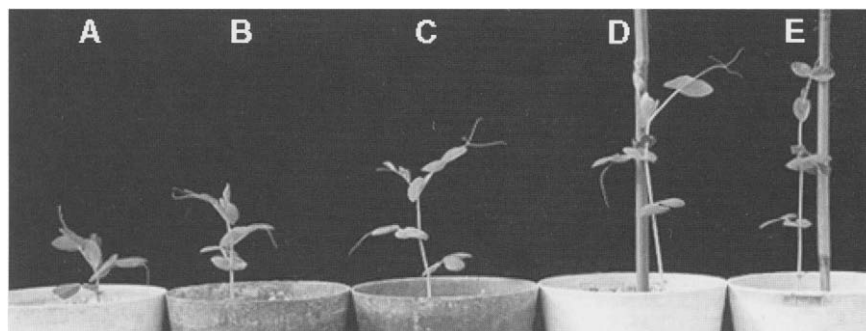


FIGURE 7-4 Elongation growth of pea (*Pisum sativum*) seedlings of dwarf (*le le*) and wild (*LeLe*) phenotypes. GA_3 was applied to epicotyl hook of dwarf pea seedlings emerging from soil at day 7 after planting, in concentrations from (left to right, A–D) 0, 1.0, 5.0, 25.0 ng per plant. A plant of the normal wild type is shown on the extreme right (E). Photo taken 14 days after planting. Courtesy William Proebsting.

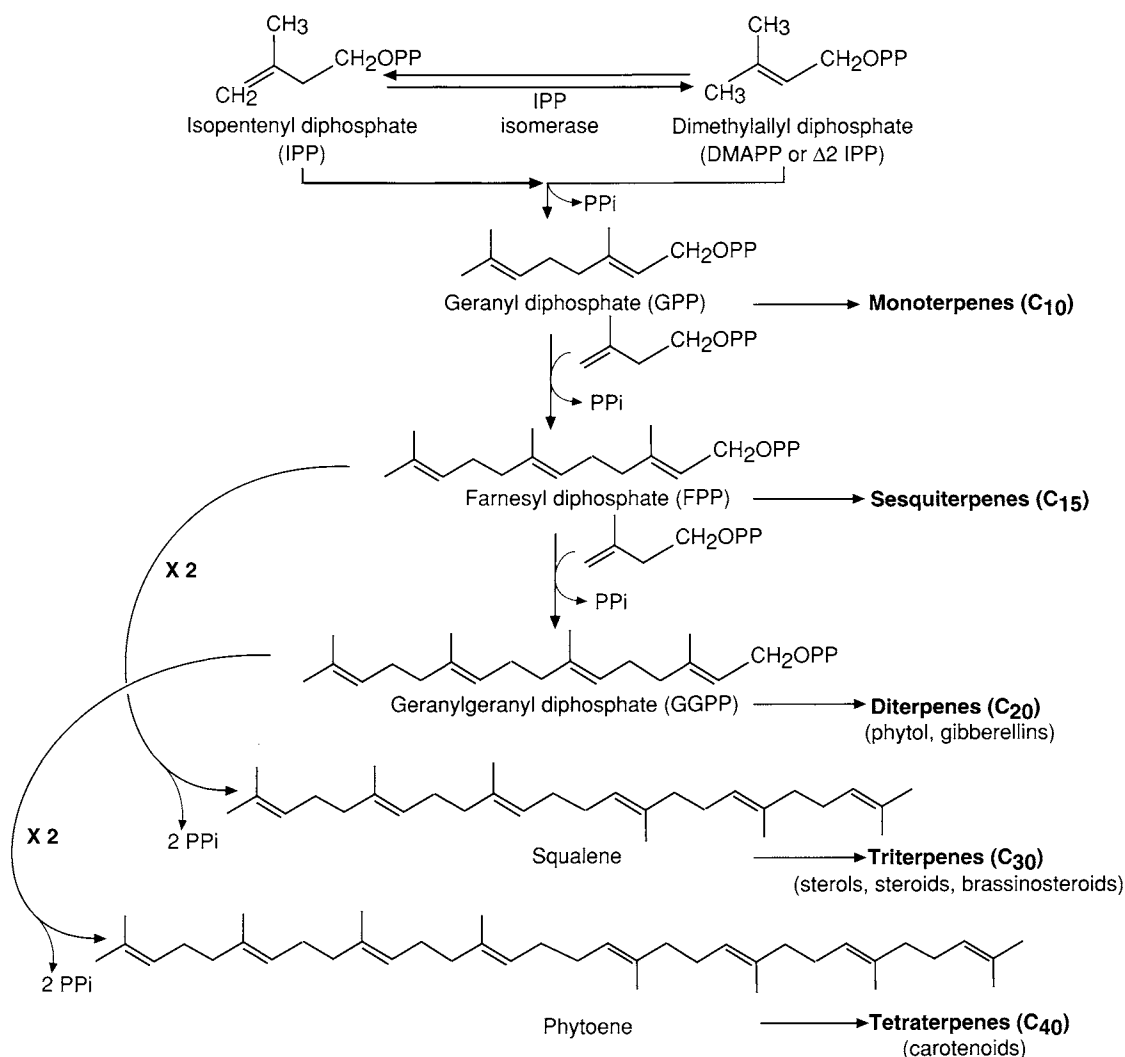


FIGURE 7-5 The terpenoid pathway. The terpenoid pathway begins with the condensation of isopentenyl diphosphate (IPP) with dimethylallyl diphosphate (DMAPP) to form the first C_{10} monoterpene, geranyl diphosphate (GPP). Each successive condensation of the isoprene unit from IPP yields successively farnesyl diphosphate (FPP), a precursor of sesquiterpenes, and then geranylgeranyl diphosphate (GGPP), a precursor of diterpenes. Dimerizations of FPP and GGPP, respectively, give rise to triterpenes and tetraterpenes. GPP, FPP, and GGPP are written in a linear form, but they can be written in a cyclized form as well. Note: In many texts, diphosphates are listed as pyrophosphates. Adapted from Taiz and Zeiger (1998).

carotenoids, which serve as accessory pigments in photosynthesis and as precursors of ABA.

4.1 Origin of IPP

Mevalonic acid (MVA) has long been regarded as the immediate precursor of IPP for all terpenoid biosynthesis. But IPP is also synthesized via a newly discovered MVA-independent pathway that utilizes pyruvate and glyceraldehyde-3-phosphate (Fig. 7-6). This latter pathway occurs primarily in the plastids (and mitochondria), whereas the former pathway occurs in the cytosol. As shown later, the early steps in GA biosynthesis from GGPP occur in plastids; hence, it is suspected that the IPP used in GA biosynthesis also comes from pyruvate and glyceraldehyde-3-phosphate, although conclusive proof is lacking.

5. BIOSYNTHESIS OF GAs

Given the large number of endogenous GAs, and GA metabolites, it was a formidable task to decide which GAs were derived from what, which were active, and which were inactive or storage forms. Nonetheless, the use of *in vitro* cell-free systems and of mutants deficient in GAs and GA synthesis inhibitors led to elucidation of the biosynthetic pathway, so much so that it is one of the best-studied biosynthetic pathways in plants. The unraveling of this pathway is a tribute to the efforts of many scientists, including Jan Graebe (Germany), Jake MacMillan (Bristol University, UK), Bernard Phinney (UCLA), Nobutaka Takahashi (University of Tokyo), Jan Zeevaert (MSU-DOE, MI), and their collaborators.

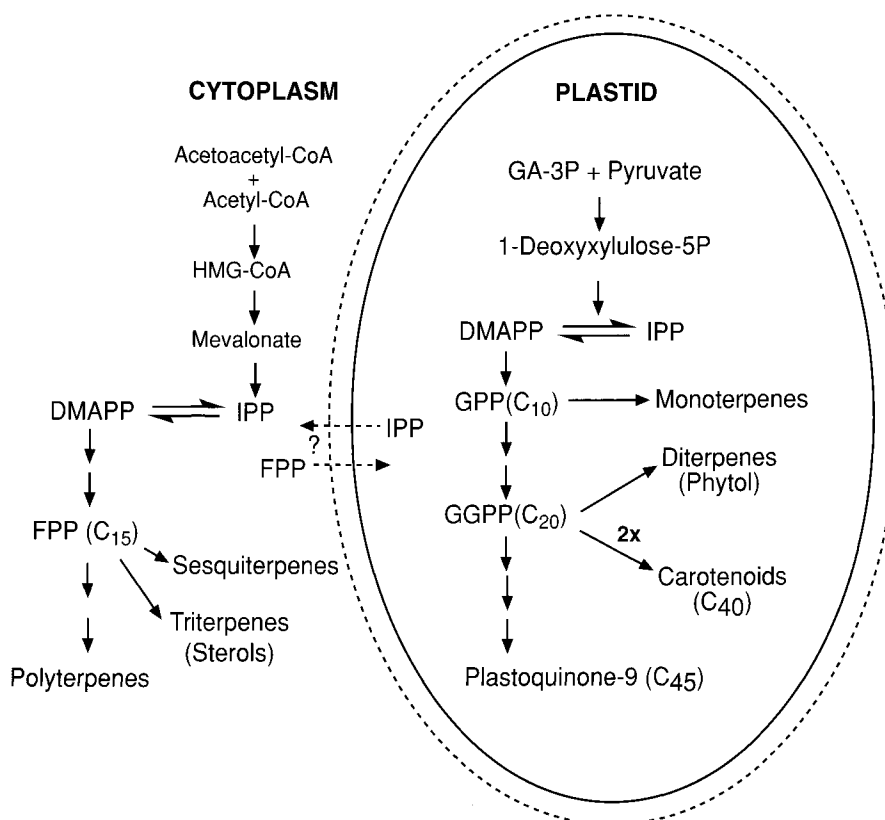


FIGURE 7-6 Two independent pathways for the synthesis of isopentenyl diphosphate (IPP). The classical pathway, which occurs in the cytoplasm, starts with the condensation of acetoacetyl-CoA and acetyl-CoA to yield hydroxymethylglutaryl-CoA (HMG-CoA). HMG-CoA reductase, a highly conserved cytosolic enzyme, catalyzes the conversion of HMG-CoA to mevalonic acid (MVA). MVA, a C₆ compound, by a series of reactions involving phosphorylations and a decarboxylation, gives rise to the C₅ IPP. The IPP derived in the cytosol is chiefly responsible for the synthesis of FPP and triterpenes, such as sterols, which are important components of cellular membranes and are also used for the synthesis of brassinosteroids. Another pathway that occurs in plastids proceeds from the condensation of glyceraldehyde-3-phosphate (GA-3P) and pyruvate (actually a C₂ derivative) to give rise to 1-deoxyxylulose phosphate and then to IPP. This pathway is proposed to give rise to all plastidic terpenes, including GGPP, phytol chain for chlorophylls, carotenoids, and related compounds. The MVA-independent pathway also occurs in mitochondria and gives rise to mitochondrial isoprene derivatives. Modified from Lichtenthaler *et al.* (1997).

Early studies on GA biosynthesis were carried out using immature, developing fruits or seeds because they were known to accumulate abundant quantities of endogenous GAs. These studies used radiolabeled precursors and followed their incorporation into different compounds *in vivo*. It was discovered later, that crude extracts from seeds, which were clear of cellular debris, i.e., cell-free systems, were still capable of GA biosynthesis from labeled precursors *in vitro*—they contained all the enzymes and cofactors necessary for GA biosynthesis. Immature seeds of wild cucumber (*Marah macrocarpa*),

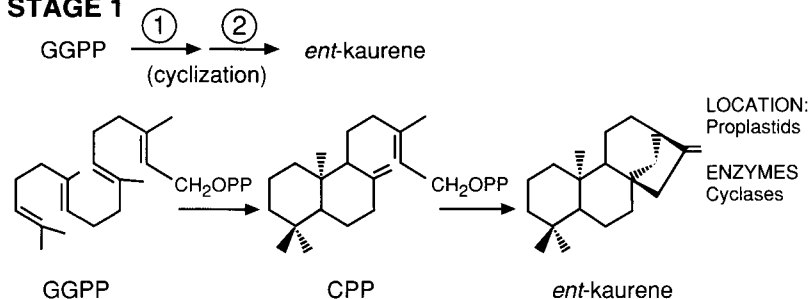
pumpkin (*Cucurbita maxima*), and pea (*Pisum sativum*) proved particularly useful. Use of an *in vitro* synthesis system, coupled with the enormous analytical capabilities of fractionation by HPLC and identification of compounds by GC and GC-MS (see Chapter 5, Box 5-1), has been a major achievement of GA research.

5.1. Stages in GA Biosynthesis

Starting from GGPP, the synthesis of GAs is divided into three stages (Fig. 7-7). The first and second stages,

GA Biosynthesis

STAGE 1



STAGE 2

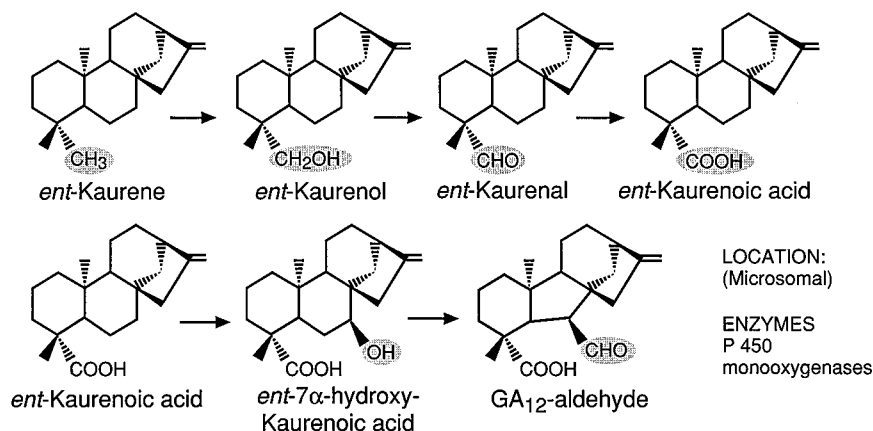


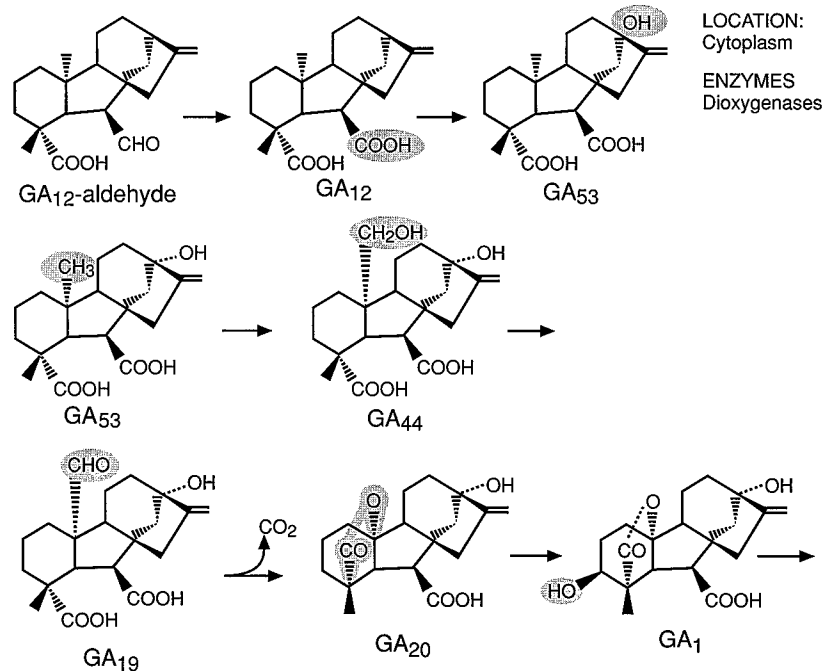
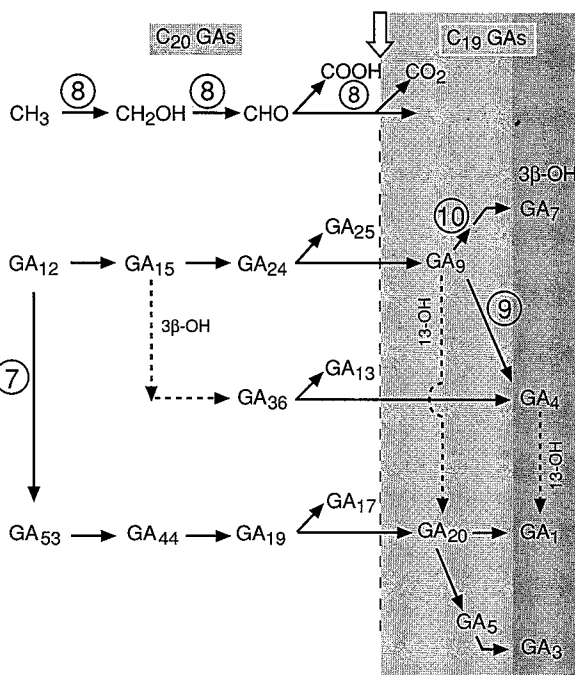
FIGURE 7-7 Stages in GA biosynthesis. Stage 1: Cyclization of GGPP *via* copalyl diphosphate (CPP) to form *ent*-kaurene. Stage 2: A series of oxidations converting *ent*-kaurene to GA₁₂-aldehyde. Stage 3: The early 13-hydroxylation and non-13-hydroxylation pathways and cross connections between them. The structures of intermediates (lower half) are shown only for the early 13-hydroxylation pathway from GA₁₂-aldehyde to GA₁. In stages 2 and 3, the modifications taking place at each step are shaded. For each stage, the class of the enzymes and their cellular locations are indicated. The important enzymes/enzyme classes are listed by numbers: (1) copalyl diphosphate synthase (CPS); (2) *ent*-kaurene synthase (KS); (3) *ent*-kaurene oxidase; (4) *ent*-kaurenoic acid hydroxylase; (5) GA₁₂-aldehyde synthase; (6) C-7 oxidase; (7) 13-hydroxylase; (8) GA 20-oxidases; (9) 3 β -hydroxylases; and (10) Δ 2,3 desaturases. Adapted from Kende and Graebe (1987), and Zeevaart (1997).

(i) $\text{GA}_{12}\text{-ald} \xrightarrow{\textcircled{6}} \text{GA}_{12}$
oxidation of C-7 to COOH

(i) $\text{GA}_{12}\text{-ald} \xrightarrow{\textcircled{6}} \text{GA}_{12}$
oxidation of C-7 to COOH

lactone formation

(ii) Oxidation of C-20



5.1.1. Stage 1

In stage 1, GGPP undergoes cyclization in two closely linked steps to give rise to the first fully cyclized

compound, *ent*-kaurene (see stage 1, Fig. 7-7). Two enzymes sequentially catalyze this reaction—copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS)—and commit GGPP on the pathway to GA biosynthesis. These enzymes occur in plastids.

5.1.2. Stage 2

In stage 2, a series of oxidations at C-19 lead to the formation of *ent*-kaurenoic acid, which is hydroxylated at C-7 to yield *ent*-7 α -hydroxykaurenoic acid. This latter compound yields GA₁₂-aldehyde by contraction of the B ring and a further oxidation at C-6 (see stage 2, Fig. 7-7). Enzymes involved in these oxidations, *ent*-kaurene oxidase, *ent*-kaurenoic acid hydroxylase, and GA₁₂-aldehyde synthase, are membrane-bound cytochrome P450 monooxygenases with a heme prosthetic group and a requirement for NADPH and O₂. In *in vitro* experiments, enzyme activity is found in microsomal fractions, and enzymes are believed to be located on the endoplasmic reticulum.

5.1.3. Stage 3

From GA₁₂-aldehyde, the favored pathways are different in different groups of plants and lead to a variety of different GAs (stage 3, Fig. 7-7). The common motifs are:

- i. Further oxidation of GA₁₂-aldehyde to give rise to GA₁₂.
- ii. Further oxidations at C-20, from CH₃ → CH₂OH → CHO, leading to lactone formation between C-19 and C-10 with elimination of CO₂. Lactone formation distinguishes C₁₉-GAs from C₂₀-GAs. Associated with lactone formation, a side reaction also produces small amounts of C₂₀-tricarboxylic GAs (e.g., GA₂₅).
- iii. In legumes and maize, probably in all plants to some extent, GA₁₂ is hydroxylated at C-13 to give rise to GA₅₃. GA₅₃ then undergoes oxidations at C-20, leading to the formation of lactone and GA₂₀. The latter undergoes 3 β -hydroxylation to give rise to GA₁. This pathway has been named the early 13-hydroxylation pathway (see stage 3, Fig. 7-7). In other plants, such as cucurbits, early 13-hydroxylation is very minor. Instead, GA₁₂, by a parallel route, gives rise to GA₉ and then GA₄. GA₃ and GA₇, both with a Δ 2,3 unsaturation, are derived from GA₂₀ and GA₉, respectively. These pathways are not rigid; both may occur in the same plant, e.g., *Arabidopsis*, and cross connections between them, involving 3 β - or 13-hydroxylation, are common. As to which route in the grid is actually favored probably is

under developmental control and provides the redundancy required in the synthesis of such an important hormone.

The early reactions in stage 3, namely C-7 oxidation of GA₁₂-aldehyde to GA₁₂ and 13-hydroxylation of GA₁₂ to GA₅₃, are likely carried out by cytochrome P450 monooxygenases. However, the subsequent oxidations at C-20, the 2,3 desaturations, and 3 β -hydroxylations (as well as the 2 β -hydroxylations that inactivate bioactive GAs, see Section 10.3) are catalyzed by dioxygenases with a specific requirement for 2-oxoglutarate and O₂ as cosubstrates. Other cofactors required are nonheme Fe²⁺ and ascorbate. In *in vitro* experiments, these enzymes appear in the soluble fraction, and hence are believed to occur free in the cytoplasm.

5.2. GA Synthesis Mutants

Many naturally occurring and, in recent years, artificially created GA synthesis mutants are known where one or another step in the GA biosynthesis is blocked. Such mutants show a dwarf habit and are well known for several crop, vegetable, or horticultural plants. Dwarf mutants in maize, pea, tomato, and *Arabidopsis* have been particularly well studied. Figure 7-8 shows the better known mutants and the steps at which GA biosynthesis is blocked.

Synthesis mutants have been very valuable in the elucidation of some steps in the GA biosynthetic pathway and in determination as to which GAs are biologically active. Thus, GA₂₀ shows high activity in bioassays, but it does so by conversion to GA₁. Evidence comes from an analysis of endogenous GAs in shoot tissues of *le* pea (shown in Fig. 7-4) and its wild-type counterpart. The mutant tissues produce very little or no GA₁ and instead accumulate GA₂₀ and its metabolites, GA₂₉ and GA₂₉-catabolite (see Section 6.3). The tissues of wild-type *LE* pea, in contrast, produce GA₁ and its metabolite GA₈, while also producing smaller amounts of GA₂₉ and GA₂₉-catabolite. Moreover, the dwarf mutant responds to exogenous GA₁ and elongates, but does not respond to GA₂₀. These data show clearly that in shoot tissues of the *le* mutant the conversion of GA₂₀ to GA₁ by 3 β -hydroxylase is impaired (Fig. 7-9). Similar data are available for *d1* maize and its wild type.

5.3. Inhibitors of GA Biosynthesis

Several synthetic compounds, known as growth retardants, inhibit stem elongation by inhibiting GA

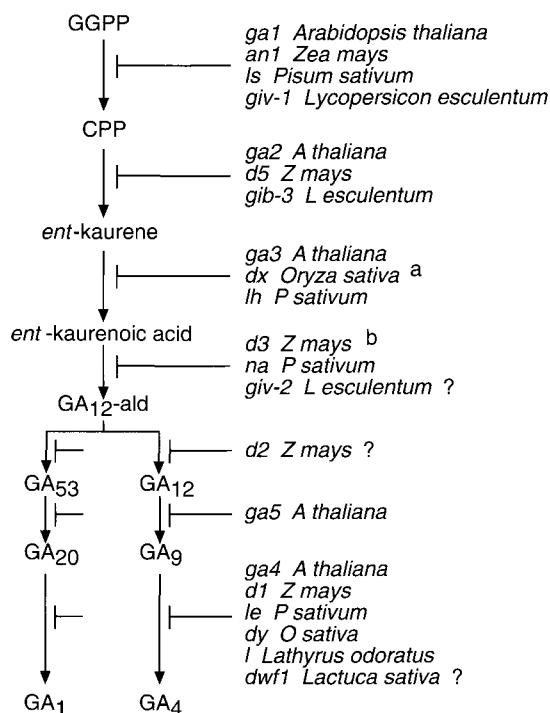


FIGURE 7-8 GA synthesis mutants and the steps at which they are blocked. These are single gene recessives in a homozygous state. A 'question mark' indicates that the position of the lesion is uncertain. It is also uncertain whether *d2* and *d3* maize are distinct mutations. [Modified and updated from Reid (1993). ^aFrom Ogawa *et al.* (1996). ^bFrom Winkler and Helentjaris (1995). As explained in Appendix 1, a mutant is written in lowercase and is italicized, e.g., *ga1*; the gene is written in capital letters and italicized, e.g., *GA1*; and the gene product, the protein, is written in capital letters, but is not italicized, e.g., GA1.

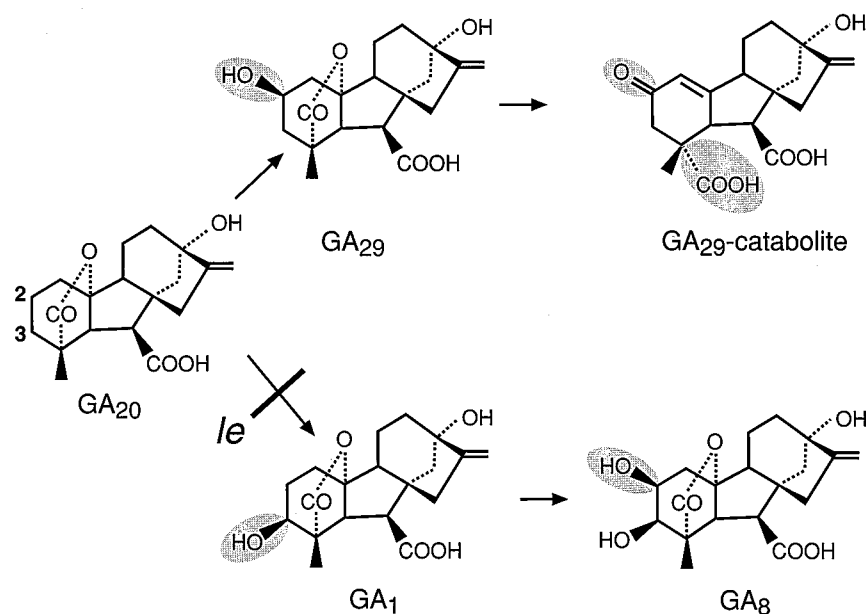


FIGURE 7-9 The effect of *le* mutation on metabolism of GA₂₀ in shoot tissue of *Pisum sativum*. The mutant is deficient in GA₁ and GA₈, but accumulates GA₂₉ and GA₂₉ catabolite. From Ingram *et al.* (1984).

synthesis. For instance, application of LAB150 978 inhibits the growth of young pumpkin seedlings, an inhibition that is overcome by a simultaneous application of GA₄ (Fig. 7-10).

GA synthesis inhibitors fall into three classes, each class specific for the enzymes catalyzing the steps in each of the three stages of GA synthesis (Fig. 7-11). The onium class of compounds, such as quaternary ammonium (e.g., chlormequat chloride or CCC, mepiquat chloride, and AMO-1618) and phosphonium (e.g., chlorphonium chloride) compounds, blocks the synthesis of *ent*-kaurene from GGPP. AMO-1618 and CCC specifically inhibit the activity of copalyl diphosphate synthase and, to a lesser extent, that of KS. The second class consists of nitrogen-containing heterocyclic compounds, such as ancymidol (a pyrimidine), tetracyclis (a norbornanodiazetidine), and triazole-type compounds (e.g., paclobutrazol, uniconazole). These compounds inhibit the oxidation of *ent*-kaurene to *ent*-kaurenoic acid by P450 monooxygenases. The third group includes acylcyclohexanediones, which inhibit the 2-oxoglutarate-dependent dioxygenases in stage 3 of GA biosynthesis. Acylcyclohexanediones, such as prohexadione-Ca and trinexapac-ethyl (a salt and an ester, respectively), are structurally similar to 2-oxoglutarate and are thought to inhibit dioxygenase activity by competing for the binding site for the cosubstrate, 2-oxoglutarate.

GA synthesis inhibitors are useful in elucidation and/or confirmation of specific steps in the GA biosynthetic pathway, especially in those plants where suitable genetic mutants are not available (e.g., wheat, bean, pumpkin). Thus, an acylcyclohexanedione, BX-112, which inhibits 3 β -hydroxylase, can be used to check which GAs are active in stem elongation.



FIGURE 7-10 Three-week-old pumpkin seedlings treated with GA₄ and/or the GA synthesis inhibitor LAB150 978 (an experimental triazole). Treatment from left to right: control, GA₄ (1 μ M), LAB150 978 (1 μ M), and GA₄ plus LAB150 978 (both 1 μ M). From Lange (1998).

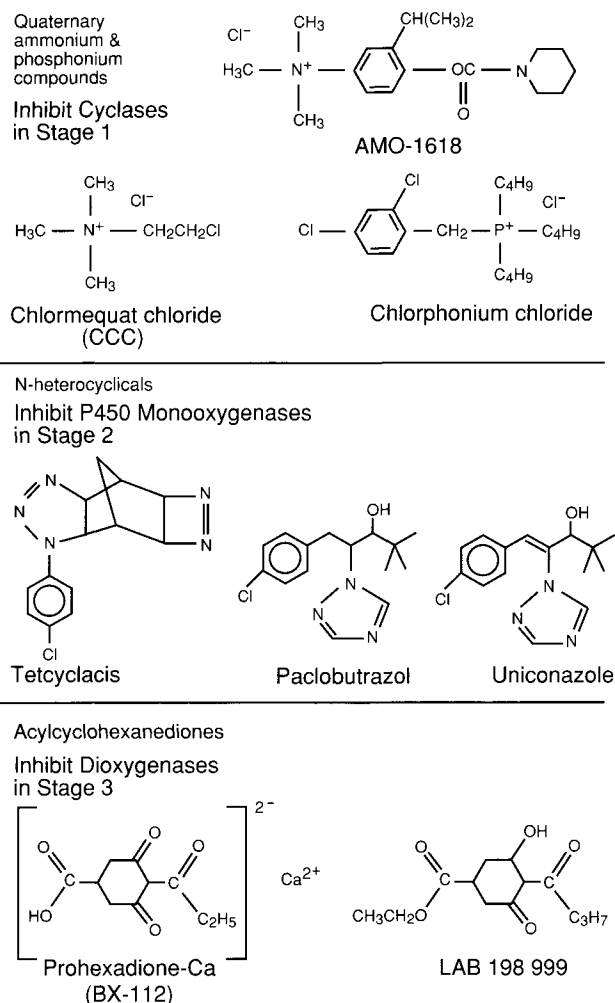


FIGURE 7-11 Representative inhibitors of GA biosynthesis. Based on Rademacher (2000). Chemical names of the inhibitors are as follows: **AMO-1618**, 2'-isopropyl-4'-(trimethylammonium chloride)-5'-methylphenylpiperidine-1-carboxylate; chlormequat chloride, (2-chloroethyl)-trimethyl-ammonium chloride [chlorocholine chloride, CCC]; chlorphonium chloride, tributyl-(2,4-dichlorobenzyl)phosphonium chloride; tetracyclis, 5-(4-chlorophenyl)-3,4,5,9,10-pentaazatetracyclo-[5.4.10^{2,6,0},11]-dodeca-3,9-diene [LAB 102 883, BAS 106 W, TCY]; paclobutrazol, (2*RS*, 3*RS*)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1*H*-1,2,4-triazol-1-yl)pentan-3-ol [PP333, PBZ]; uniconazole-P, (E)-(R*S*)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1*H*-1,2,4-triazol-1-yl)pent-1-en-3-ol [S-3307D, XE-1019, UCZ]; prohexadione-calcium, calcium 3,5-dioxo-4-propionylcyclohexanecarboxylic acid [BX-112]; and LAB 198 999 (an experimental compound), 4-(*n* propyl- α -hydroxymethylene)-3,5-dioxocyclohexanecarboxylic acid ethyl ester. Commercial names are given within brackets.

If GA₂₀, GA₉, or GA₁ is supplied along with prohexadione-Ca, it can be shown readily that only GA₁ is active in promoting stem elongation, whereas others are not because they cannot be 3 β -hydroxylated to GA₁ or GA₄, respectively.

TABLE 7-1 cDNAs and Genes Encoding Enzymes Involved in GA Synthesis^a

Stage	cDNA (—) and Gene	Enzyme	Plant
1	<i>GA1</i>	Copalyl diphosphate synthase	<i>Arabidopsis thaliana</i>
	<i>An-1</i>	Copalyl diphosphate synthase	<i>Zea mays</i>
	<i>LS</i>	Copalyl diphosphate synthase	<i>Pisum sativum</i>
	—	<i>ent</i> -Kaurene synthase	<i>Cucurbita maxima</i>
	<i>GA2</i>	<i>ent</i> -Kaurene synthase	<i>A. thaliana</i>
2	<i>D3</i>	A P450 monooxygenase	<i>Z. mays</i>
	<i>GA3</i>	<i>ent</i> -Kaurene oxidase	<i>Arabidopsis</i>
3	—	GA 20-oxidase	<i>C. maxima</i>
	<i>GA5</i>	GA 20-oxidase	<i>A. thaliana</i>
	—	GA 20-oxidase	<i>Spinacea oleracea</i>
	—	GA 20-oxidase	<i>P. sativum</i>
	—	GA 20-oxidase	<i>Phaseolus vulgaris</i>
	—	GA 20-oxidase	<i>Oryza sativa</i>
	—	GA 20-oxidase	<i>Marah macrocarpus</i>
	<i>Ls20ox1</i> and <i>Ls20ox2</i>	GA 20-oxidases	<i>Lactuca sativa</i>
	<i>GA4</i> and <i>GA4H</i>	3 β -Hydroxylases	<i>A. thaliana</i>
	<i>LE</i>	3 β -Hydroxylase	<i>P. sativum</i>
	<i>Ls3h1</i> and <i>Ls3h2</i>	3 β -Hydroxylases	<i>L. sativa</i> ^b

^aAll data are from Hedden and Kamiya (1997) and Hedden and Proebsting (1999).

^bFrom Toyomasu *et al.* (1998).

It is important to note, however, that these compounds inhibit the activities of general classes of enzymes (e.g., monooxygenases, dioxygenases); hence, they are not exclusively GA synthesis inhibitors. For instance, P450 monooxygenases are a large family of enzymes that catalyze oxidations in many other metabolic pathways besides GA biosynthesis, and it is possible that distinct triazole-type compounds may inhibit specific isoforms of the enzyme. Triazoles are known to affect the metabolism of ABA as well as brassinosteroids.

As growth retardants, these compounds are used commercially in agriculture and horticulture industries. For instance, chlormequat and mepiquat chloride are used as antilodging agents in cereal crops and to reduce excessive vegetative growth in cotton. The acylcyclohexanediones, trinexapac-ethyl and prohexadione-Ca, are used for stem stabilization in cereal crops and oilseed rape, growth control of turf grass, and reduction of vegetative growth in fruit trees.

5.4. Enzymes of GA Metabolism and Their Genes

In recent years, cDNAs and genes encoding most of the major enzymes in GA biosynthesis have been cloned using a variety of techniques (Table 7-1). Some have been cloned from purified proteins and DNA probes based on amino acid sequences, some from known mutants in GA biosynthesis, and some have no known mutants, but have been cloned using homology-based techniques. The availability of these clones has opened the way for determining whether different isoforms of the same enzyme exist, their organ- and tissue-specific localization, and their relative abundance. This information is crucial to understanding sites of GA biosynthesis and developmental and environmental factors that regulate such synthesis (see Sections 5 and 6.1).

As shown in Table 7-1, several GA 20-oxidase cDNAs have been cloned. Sequence comparisons of these cDNAs and their functional expression in

Escheichia coli suggest that these enzymes are encoded by a large family of genes with multiple members present in the same plant. The different isoforms produce different products and differ in tissue/organ specificity. For example, most clones, when expressed in *E. coli*, produce enzymes that convert GA₁₂ or GA₅₃ (C₂₀-GAs) to GA₉ or GA₂₀ (C₁₉-GAs), respectively; the clone from pumpkin (*Cucurbita maxima*) designated P16, given the same C₂₀ precursors, produces primarily tricarboxylic GAs and little C₁₉-GAs. Three different cDNAs, representing three different genes for GA 20-oxidases, are known from *Arabidopsis*. While all show a substrate preference for GA₁₂ over GA₅₃, their transcripts are expressed differentially in an organ-specific manner (Fig. 7-12).

Several cDNA clones and genes encoding different isoforms of 3 β -hydroxylases are also known from *Arabidopsis*, pea, and lettuce; and different isoforms are expressed in an organ-specific manner.

In contrast to the abundance of genes encoding GA 20-oxidases and 3 β -hydroxylases in the same plant, the number of genes encoding the copalyl diphosphate synthase, *ent*-kaurene synthase, and *ent*-kaurene oxidase seems to be limited. For instance, in *Arabidopsis*, genomic analysis has so far indicated only one gene encoding each of these enzymes, and these genes are expressed in a development-specific manner. While other explanations are possible, this suggests a tighter regulation of GA biosynthesis in earlier stages and the possibility of environmental and/or organ/

tissue-specific regulation of GA synthesis in stage 3. An interesting example of organ-specific regulation of a 3 β -hydroxylase gene is provided by the *le* mutation in pea. As explained earlier, *le* pea is deficient in GA₁ in shoot tissue and shows dwarfism, but the GA content in roots and root growth are normal. In marked contrast, the *ls*, *lh*, and *na* mutants of pea, which are impaired in enzymes involved in stages 1 and 2 of GA biosynthesis (see Fig. 7-8) are deficient in GAs both in shoot and in root.

5.5. Organ and Subcellular Sites of GA Biosynthesis

GA biosynthesis has long been thought to occur in young parts of a plant, shoot apices, young leaves, developing fruits and seeds, and young roots; in contrast, mature leaves and stems are believed to be relatively deficient in this synthesis. These assumptions, based on bioassays and the capacity of different plant tissues to incorporate radiolabeled precursors into GAs, are supported by expression patterns of GA1 and GA3 genes in *Arabidopsis*. Both genes are expressed predominantly in young meristematic areas in root and shoot tips, procambial or cambial tissues, elongating stems, and floral parts; their expression declines in older mature plants and in rosette leaves.

As to subcellular sites of synthesis, there is increasing evidence that the conversion of GGPP to *ent*-kaurene occurs in plastids. In one study, different cell fractions from pea and wheat plants were used for the incorporation of labeled GGPP into *ent*-kaurene. Maximal incorporation was found in proplastid or leucoplast fractions from shoot apices, intercalary meristems, and young endosperm tissue in seeds, which are all areas of rapid cell division and growth. Incorporation was not seen in differentiated chloroplasts from mature leaves. Moreover, the GA1 gene encodes a protein with a signal peptide that targets it to the plastids. Such location of *ent*-kaurene synthesis is in agreement with the postulated synthesis of IPP and GGPP in plastids (see Section 4, Fig. 7-6).

As mentioned earlier, stages 2 and 3 of GA biosynthesis are catalyzed by ER-bound P450 monooxygenases and by dioxygenases in the cytosol, but how *ent*-kaurene enters the cytoplasm is not clear. It is also not clear whether any of the different steps are compartmented in any way or whether any of the intermediate GAs are translocated long distance in the phloem stream to other parts of the plant.

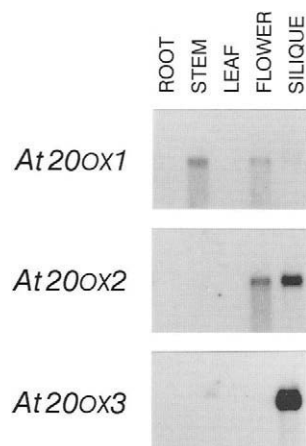


FIGURE 7-12 Expression patterns of three GA 20-oxidases in *Arabidopsis thaliana* (wild type, *Landsberg erecta*) plants. Northern hybridization of cDNA clones *At20ox1*, *At20ox2*, and *At20ox3* to RNA extracted from roots, stems, expanded leaves, inflorescences, and developing siliques. One microgram of poly (A)⁺ RNA was loaded in each lane. From Phillips *et al.* (1995); note: cDNA clones have been renamed.

6. REGULATION OF GA LEVELS IN THE PLANT

The concentrations of active GAs, such as GA₁ or GA₄, are precisely regulated in plant tissues by a regulation of their synthesis and their inactivation by conjugation and/or catabolic breakdown. GAs may also be transported to other parts of the plant or may be reversibly inactivated and stored in some compartment, such as vacuoles, and reused later.

6.1. Regulation of Synthesis

The GA biosynthetic pathway involving multiple steps catalyzed by different types of enzymes provides many opportunities for regulatory control. The enzymes may be turned on by developmental or environmental cues or may be inhibited by some feedback control. Our information on these topics is still limited, but with the cloning of genes and cDNAs for enzymes, it is beginning to accumulate. It appears that GA synthesis is regulated at least at four steps: (i) synthesis of *ent*-kaurene in stage 1; (ii) oxidation of *ent*-kaurene in stage 2; (iii) modulation of the activities of the GA 20-oxidases leading to the formation of C₁₉ GAs; and (iv) 3 β -hydroxylations that give rise to biologically active C₁₉-GAs (e.g., GA₁, GA₄), both in stage 3. Yet another way to regulate the levels of active GAs is to regulate their inactivation by 2 β -hydroxylation (see Section 6.3).

6.1.1. Regulation of Synthesis of *ent*-kaurene

GGPP is abundant in plant tissues, and it would be expected that the first step in *ent*-kaurene synthesis, namely the commitment of GGPP to CPP catalyzed by coplayl diphosphate synthase, is strictly regulated. This is supported by two observations: (i) the enzyme CPS occurs in very low abundance in plant tissues and (ii) CPS transcripts also occur in low abundance and are expressed only in young, growing tissues, whereas KS transcripts are abundant and constitutively present in nearly every organ.

6.1.2. Environmental Regulation of GA Synthesis

Environmental factors, especially temperature and light, are known to affect GA levels and/or sensitivity of the plant response to GAs. Such regulation is known for enzymes in both stages 2 and 3 of GA biosynthesis. Thus, in *Thlaspi arvense* (field pennycress), an exposure to cold temperature (2–10°C for several days) followed by a return to normal temperature (21°C) induces stem extension growth and

flowering. These effects can be obtained by application of GAs without exposure to cold temperature. In non-induced plants, *ent*-kaurenoic acid accumulates in the shoot tips, which are the presumed site of perception of cold temperature. In induced plants, however, the levels of this intermediate fall to low levels within days with attendant formation of the biologically active GAs. Moreover, microsomal fractions from cold-induced plants metabolize *ent*-kaurenoic acid and *ent*-kaurene to much greater levels than the microsomal fractions from noninduced plants, which suggests a change in the level or activity of a P450 monooxygenase.

In many rosette plants that require long days (LDs) for flowering (they remain vegetative under short day conditions), the induction of flowering is accompanied by rapid stem elongation (bolting) and is mediated by GAs. Many studies indicate that the main effect of the photoperiod is on the synthesis/activity of GA 20-oxidases. In spinach (*Spinacea oleracea*), an obligate long-day plant, transition of plants from short days (SDs) to long days results in bolting and accumulation of GA₂₀ and GA₁. Northern blot analysis indicates that the level of GA 20-oxidase mRNA is higher in plants in LD than in SD conditions, with the highest level of expression in the shoot tips and elongating stems (Fig. 7-13). Similar results are reported for bolting and flowering in *Arabidopsis*, a facultative long-day plant.

There are many reports about the effects of light, especially red light, on stem elongation, as well as on seed germination. These effects are covered in Chapter 26.

6.1.3. Feedback Inhibition of GA Synthesis

There is considerable evidence that a bioactive GA, or some product of its activity, exercises a negative

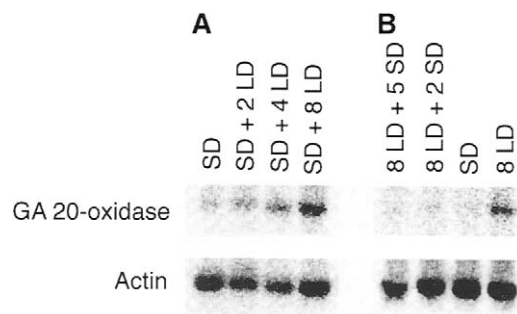


FIGURE 7-13 Levels of GA 20-oxidase mRNA in shoot tips of *Spinacea oleracea* plants kept under short days (SD), followed by long days (LD) or kept under LDs followed by SDs. Actin mRNA is the control. From Wu *et al.* (1996).

feedback control on GA synthesis itself. The primary targets for such feedback inhibition are GA 20-oxidases and 3 β -hydroxylases. Evidence comes both from measurement of endogenous GA levels and from Northern hybridizations of enzyme transcript levels. In mutants where 3 β -hydroxylation is impaired (e.g., *d1* maize, *le* pea), GA₂₀ is accumulated, but the concentration of the preceding C₂₀-GAs, such as GA₁₉, is reduced. Treatment of tall (*LE*) and dwarf (*le*) lines of pea with 2,2-dimethyl GA₄, a synthetic but highly active GA, stimulates shoot elongation, as expected, but also decreases the concentrations of GA₂₀ and GA₁, while increasing the concentration of the preceding GA₁₉ (Table 7-2). These modulations in GA₁₉ and GA₂₀ levels likely reflect changing levels of GA 20-oxidase activity.

More direct evidence comes from the *Arabidopsis* mutant, *ga1*, which is deficient in endogenous GAs because the conversion of GGPP to *ent*-kaurene is blocked. In such a background, the transcript levels of three GA 20-oxidase genes were much higher than in the wild type. However, treating mutant plants with GA₃ caused a dramatic reduction in the levels of the transcripts (Fig. 7-14). Similarly, the levels of GA₄ gene transcripts in *Arabidopsis*, encoding a 3 β -hydroxylase, are high in GA-deficient mutants, but are sharply downregulated on treatment of plants with active GAs.

Such feedback regulation is not seen for transcripts of GA₁ or GA₃ genes, which encode enzymes involved in stages 1 and 2 of GA biosynthesis. Those genes

are thought to be developmentally regulated. Although the phenomenon of feedback regulation of GA 20-oxidases or 3 β -hydroxylases by an active GA (or a GA-induced product) is well recognized, the actual mechanism by which it is brought about is unknown.

6.2. GA Conjugates

Certain parts of the GA molecule are essential for biological activity in higher plants. These include the -COOH group at C-6 and the presence of the lactone group. In addition, the presence of 3 β -OH is believed to be essential for stem elongation, α -amylase synthesis in cereal aleurone tissue, and some other responses, although not for all.

Conjugation of the C-6-COOH group with a sugar (usually glucose) renders a GA inactive (Fig. 7-15A). Such conjugated GAs, referred to as GA-glucosyl esters, occur naturally, and their formation is reversible. GAs are stored in maturing seeds of many plants as glucosyl esters, and their hydrolysis may provide limited amounts of free GA during early seedling growth. They are also synthesized in vegetative tissues, especially if active GAs are fed to plant tissues/organs in unnaturally large amounts.

The addition of a sugar moiety to a GA molecule renders it more polar than the parent GA, and it is believed that GAs are stored in vacuoles in this form. They may also be translocated a long distance in the phloem tissue in a conjugated form, although there are many reports of free GAs being transported in the phloem stream as well.

6.3. Irreversible Deactivation

The most common method and probably the most important for deactivation of biologically active GAs or their precursors is by 2 β -hydroxylation (Fig. 7-15B). Thus, 2 β -hydroxylation of GA₁ leads to formation of the inactive GA₈, of GA₄ to the inactive GA₃₄, and of GA₂₀ to GA₂₉. The hydroxyl group at C-2 may undergo further oxidation with a concomitant breakdown of lactone to form a catabolite, e.g., GA₈ catabolite or GA₂₉ catabolite. The formation of 2 β -hydroxylated GAs and their catabolites is an essentially irreversible process, and the products are the end products of GA catabolism. That 2 β -hydroxylation is essential for regulating the concentration of endogenous GAs involved in stem elongation (e.g., GA₁ or GA₄ or their precursors, GA₂₀ or GA₉) is shown by the *slender* mutant in pea. The mutant is defective in 2 β -hydroxylation and the plants grow to be inordinately tall

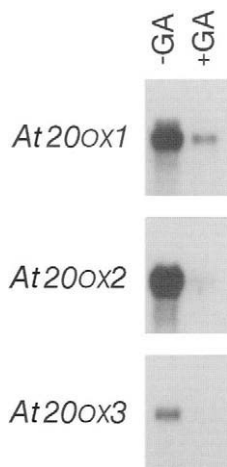
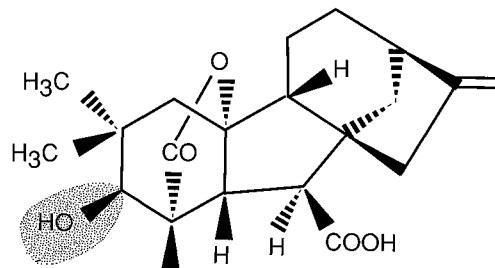


FIGURE 7-14 Expression of three cDNAs encoding GA 20-oxidases in a GA-deficient mutant (*ga1-2*) of *Arabidopsis*. poly(A)⁺ RNA (mRNA) was extracted 16 h after spraying the plants with 100 μ M GA₃ or with water (control, no GA). For each hybridization, 1 μ g poly(A)⁺ RNA was loaded and hybridized to three cDNA clones, *At20ox1*, *At20ox2*, and *At20ox3*. From Phillips *et al.* (1995).

TABLE 7-2 Contents of GA₁₉, GA₂₀, and GA₁ in Expanding Tissues of Dwarf (*Na le*) and Tall (*Na Le*) Genotypes of *Pisum sativum*^a



2,2-dimethyl GA₄

Phenotype (genotype)	Height (cm) ± SE	GA ₁₉	GA ₂₀ (ng · g fw ⁻¹)	GA ₁
Dwarf (<i>Na le</i>)	10.0 ± 0.3	0.6	36.8	0.2
Dwarf (<i>Na le</i>) + GA	26.3	2.7	8.7	0
Tall (<i>Na Le</i>)	29.5 ± 1.7	1.3	15.7	1.9
Tall (<i>Na Le</i>) + GA	35.0 ± 1.1	2.1	7.9	0.4

^aPlants were either untreated or treated with GA (6 µg 2,2-dimethyl GA₄, 3 µg at germination, and 3 × 1 µg applications at 3-day intervals). Endogenous GAs were extracted from expanding tissues at node 6 (about 20 days from sowing) and quantified by GC-SIM. The structure of 2,2-dimethyl GA₄ is shown. Adapted from Martin *et al.* (1996).

and slender because of an overabundance of active GA₁. The wild-type gene, *SLENDER*, from pea was cloned by two independent groups and shown to encode a 2β-hydroxylase. The enzyme, which is a dioxygenase similar to GA 20-oxidases and 3β-hydroxylases, catalyzes both hydroxylation and further oxidation to the catabolites. In view of its multifunctionality, it is now known as 2-oxidase. Because 2β-hydroxylation is an important step in the maintenance of GA homeostasis in plants, the cloning of the 2β-hydroxylase gene is especially significant. There are indications that different members of the gene exist in pea and encode isoforms that show differential expression in shoot vs seed tissues. Further studies will undoubtedly clarify factors that induce their expression and characterize the enzyme. As expected, the activity of 2-oxidases, like that of dioxygenases involved in stage 3 of GA synthesis, is inhibited by growth retardants of the acylcyclohexanedione type, which, in a reverse twist, leads to enhanced levels of bioactive GAs.

GA-glucosyl ethers, where a glucose residue is linked to a OH group at C-2, C-3, or C-13, also occur naturally (Fig. 7-15C). Opinions differ as to whether the glucosyl ethers are the end products of GA catabolism. Because many of them, reported as naturally occurring, result after 2β-hydroxylation, which in itself renders GAs inactive, 2β-glucosyl ethers are

also end products of catabolism. 3β- or 13α-glucosyl ethers of active GAs, such as GA₁ or GA₃, or 3β-glucosyl ether of GA₄, however, are reported to be hydrolyzed back to the parent GA, and, thus, they may represent storage forms.

Other modifications in the GA structure include 3α-hydroxylation. 3α-OH-GAs, such as 3-*epi*-GA₁ or 3-*epi*-GA₄ (see Fig. 7-3), occur naturally in extracts of plant tissues in small amounts, but are not common. They can be prepared easily in the laboratory, however. In most bioassays, they give low biological activities. Likewise, methylation of C-6-COOH does not seem to occur naturally in higher plants. The enzyme is reported to be lacking, but artificially methylated GAs lack biological activity in higher plants. Both 3-*epi*-GAs and GAs methylated at C-6-COOH occur naturally in fern gametophytes and are active biologically (see Section 9).

Our information on enzymes catalyzing the formation or hydrolysis of glucosyl esters or ethers or that result in epimerization or methylation is scant.

7. ENDOGENOUS LEVELS

An estimate of endogenous levels of GAs in a tissue or organ is complicated by the fact that in any tissue or organ, not one, but often a dozen or more GAs are

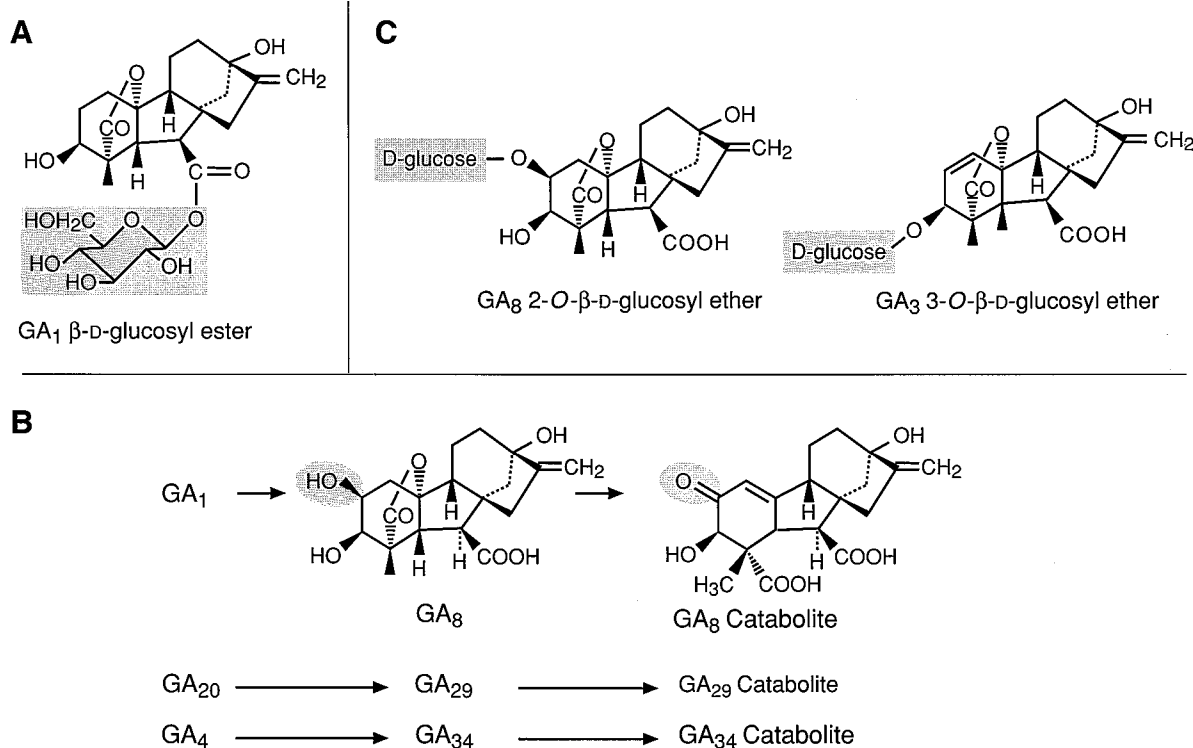


FIGURE 7-15 GA conjugation and irreversible deactivation of GA. (A) GA₁ glucosyl ester. (B) 2β-Hydroxylation of GA₁ to GA₈ and further oxidation to GA₈ catabolite. Similar inactivated GAs from GA₂₀ and GA₄ are indicated. (C) GA₈ and GA₃ glucosyl ethers.

present. Accordingly, one has to speak of the total GA content or the content of any one specific GA. The earlier quantitative estimates based on bioassays, although useful in the 1960s and 1970s, are misleading because they measured only the active GAs or GAs that were converted *in vivo* to active forms; they could not measure the inactive forms, and the results were expressed globally in terms of GA₃ equivalent. These data therefore must be interpreted with caution. Determinations of individual GAs using GC and GC-MS for qualitative analysis and quantitation or by immunoassays are now possible and provide more reliable data on endogenous contents of individual GAs.

Most recent measurements indicate that the concentration of bioactive GAs, GA₁, GA₄, and GA₃ are relatively low in vegetative tissues, such as leaves, stems, shoot apices, and cambial tissues. In many plants, GA₁ is the predominant active GA, and its concentrations are in the range of 1–10 ng · g fw⁻¹, with lesser amounts for GA₄ and GA₃. In other plants, GA₉ or GA₄ concentrations may be higher than those of GA₁. Various other GAs, precursors of active GAs or deactivated products, accumulate to varying degrees depending on the physiological state of the tissue. Developing fruits and seeds contain much larger

amounts of total GAs, in some instances up to 8–10 μg · g fw⁻¹, but most of these GAs are in conjugated forms, such as glucosyl esters, or in the form of inactive precursors, such as GA₂₀.

8. WHY ARE THERE SO MANY GAs?

A question that has interested many investigators is why are there so many different GAs. Now of course we know that only a few GAs show biological activity, others are either intermediates in biosynthesis of the active forms, or are inactive metabolites. It could be that a large number of steps involved in biosynthesis allows many regulatory points of control. We have already seen how the activities of some enzymes in the synthetic pathway may be regulated by a negative feedback control or by environmental factors, such as temperature or day length (see Section 6.1 above). Second, the large number of GAs could have something to do with taxonomic preferences. For example, results from numerous bioassays indicate that in pea epicotyl elongation or induction of α-amylase in cereal aleurone tissue, GA₁ and GA₃ show greater activity

TABLE 7-3 Biological Activities of 3,13-Hydroxylated GAs (GA_1 , GA_3) vs 3-Hydroxylated GAs (GA_4 , GA_7) in Four Bioassays^a

GA	Barley aleurone induction of α -amylase	Elongation response		
		Dwarf pea epicotyl	Cucumber hypocotyl	Arabidopsis hypocotyl
GA_1	****	***	**	***
GA_3	****	****	**	—
GA_4	***	***	***	****
GA_7	***	***	****	—
GA_8	*	*	0	0

^aComparative data for GA_8 , a 2 β -hydroxylated GA, are given also. The greater the number of asterisks, the greater the activity; — not determined. Data from Crozier and Durley (1983), except for *Arabidopsis*, which are from Zee-vart and Talon (1992).

than GA_4 or GA_7 . The opposite is true for hypocotyl elongation in both cucumber and *Arabidopsis*. In these plants, GA_4 or GA_7 have much higher activities than GA_1 or GA_3 (Table 7-3). Third, it is possible that different GAs regulate different processes. Stem elongation and the flowering response, especially in herbaceous long-day plants, in angiosperms are apparently regulated by different GAs. 3 β -Hydroxylated GAs promote stem elongation, but do poorly in the induction of flowering, whereas the reverse is true for GAs that show multiple hydroxylations in C and D rings. Cone production in different families of conifers is regulated by different GAs. Members of the pine family show an early onset of cone production by the application of $GA_{4/7}$, whereas members of the juniper family are more responsive to $GA_{1/3}$.

9. OTHER SUBSTANCES WITH GA-LIKE ACTIVITY

Some natural as well as synthetic substances show GA-like activities in bioassays. GAs are known to bring about antheridia formation in fern gametophytes. A search for antheridiogens (antheridia-promoting substances) in ferns has led to the isolation of some novel GA-like structures (Fig. 7-16). Antheridic acid is active in promoting both spore germination in the dark and in antheridia formation in the gametophytes of *Anemia* species belonging to Schizaeaceae. Antheridic acid has a lactone and C-6-COOH, but it has 3- α -OH in the A ring and has a rearranged C/D ring system. In *Lygodium* ferns, a methylated GA, GA_{73} methyl ester has high biological activity in promoting antheridia formation. In both ferns, the C_{19} -GAs from higher plants also show activity to varying extents, although usually one to two orders of magnitude less than the corresponding

methyl esters; it is possible that free GAs become active only upon methylation to the corresponding ester.

The fern system is instructive. Whereas antheridic acid and GA_{73} -Me have biological activity in gametophyte generation, sporophyte plants seem to contain the same C_{19} -GAs as angiosperms. In *Anemia phyllitides*, 6-week-old prothallia had GA_9 , GA_{24} , GA_{25} , and antheridic acid and 3-*epi*- GA_{63} , whereas the sporophytic plants had GA_4 , GA_9 (most abundant), GA_{19} , GA_{20} , GA_{24} , and GA_{25} , but neither antheridic acid nor 3-*epi*- GA_{63} was detectable. It is possible that different GAs or GA-like substances in ferns are used in a switch from a sporophytic to a gametophytic developmental program. Much more work with many other ferns needs to be done to support or disprove this assumption.

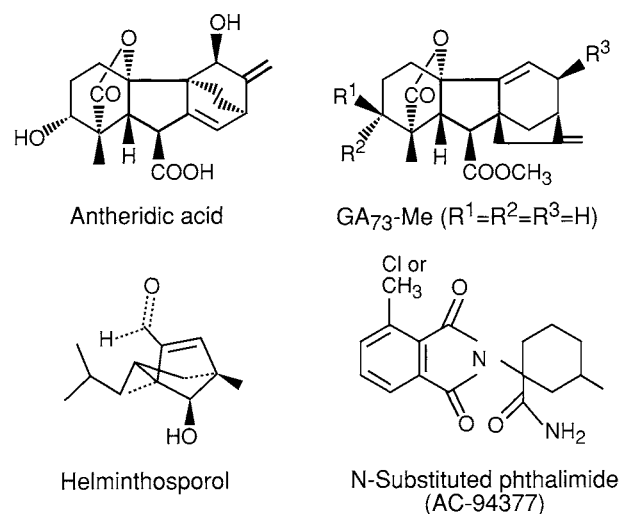


FIGURE 7-16 Structures of some GA-like substances: antheridic acid, GA_{73} Me, helminthosporol, and an N-substituted phthalimides.

Helminthosporol from *Helminthosporum*, a fungus, has activity in some bioassays; some synthetic substances, such as N-substituted phthalimides, promote stem elongation in dwarf maize (*d5*) and dwarf rice (Tanginbozu), and α -amylase synthesis in barley aleurone, but these responses have not been pursued to any extent.

10. CHAPTER SUMMARY

Gibberellins are a large class of cyclic diterpenes that occur in all vascular plants as well as in many fungi. The number of gibberellins is large, but not all are biologically active; many are intermediates in the synthesis of bioactive GAs, whereas others are inactivated forms. Active GAs serve many important roles in vascular plants, including stem/leaf/root elongation, seed germination, mobilization of food reserves in cereal grains, cambial activation, and flowering and floral development. In fern gametophytes, they are involved in spore germination, and antheridia formation. The biosynthesis of GAs occurs in three stages in three distinct subcellular locations. In the first stage, geranylgeranyl diphosphate is converted in a two-step process to *ent*-kaurene. These steps occur in plastids, and the first enzyme, copalyl diphosphate synthase, provides the committing step. In the second stage, *ent*-kaurene is translocated to the cytoplasm, where it is progressively oxidized and hydroxylated to yield GA₁₂-aldehyde by a series of ER-bound P450 monooxygenases. In the third stage, GA₁₂-aldehyde is further oxidized, hydroxylated, and, in some cases, desaturated by soluble enzymes in the cytosol to yield the bioactive C₁₉-GAs. Genes or cDNAs encoding most of the major enzymes/enzyme classes have been cloned and are providing information on sites of synthesis of GAs, as well as on the regulation of GA synthesis by environmental and/or organ/tissue-dependent factors. Also, there is evidence that active GAs regulate their own synthesis by a negative feedback inhibition of GA 20-oxidases and 3 β -hydroxylases, enzymes responsible for the production of bioactive C₁₉-GAs in the third stage. An extended GA metabolic pathway provides multiple opportunities for regulatory control over its endogenous amounts. GA homeostasis is maintained not only by the control of synthesis but also by the inactivation of bioactive GAs by conjugation and/or irreversible deactivation via 2 β -hydroxylation. The large number of GAs in vascular plants provides the possibility for functional specialization. For example, certain GAs may be more involved in stem/leaf elongation, whereas others are more involved in the flowering response. It also provides for specificity at the taxon level, i.e., different GAs are more effective, relative to each other, in bringing about the same response in two different taxonomic groups.

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Cytokinins

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1. DISCOVERY

Haberlandt, a German plant physiologist, noted as far back as 1913 that certain diffusible factors from phloem tissue, e.g., phloem exudate, could cause cell division in potato tubers. Later in 1921 he discovered that healing of cut plant tissues by cell division was prevented if the cut surfaces were washed with water. Both observations suggested the presence of a soluble factor in plant tissues that promoted cell division.

In the 1940s and 1950s, the techniques of plant tissue culture were being developed, and it was noted that callus cultures from tobacco pith explants or carrot roots, both favorite objects for study, placed on agar blocks soaked with nutrients and auxin still showed very little cell division. They required other substances, e.g., coconut milk (liquid endosperm from coconut), or extracts from vascular tissues, yeast extract, autoclaved DNA, or even adenine. In 1956, Carl Miller in Folke Skoog's laboratory at Wisconsin University, Madison, discovered that a substituted adenine, 6 furfuryl amino purine, obtained from autoclaved herring sperm DNA was far more potent than adenine in promoting cell division in tobacco pith explants. This substance was given the name **kinetin** (Fig. 8-1) [for a personal and interesting account of this discovery, see Skoog (1994)].

Kinetin does not occur naturally in plants (but see Section 8). In the search for natural substances, it was expected that endosperm tissue, which provides nutrition for the growth of embryo during its early

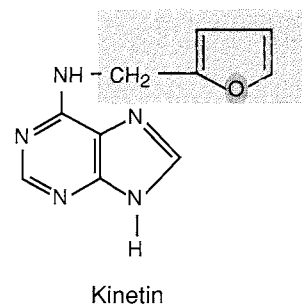


FIGURE 8-1 Kinetin or N⁶-(furfurylamino)purine. The side chain is shaded for comparison with side chains in Fig. 8-2.

development or, postgerminatively, for growth of young seedling (see Chapters 18 and 19), might be a promising material to look for hormones that promote cell division. Liquid endosperm from coconut, maize, horse chestnut, and immature fruits of banana were favorite objects for study. Thus, in 1963, D. S. Letham at CSIRO, Canberra, Australia, isolated a substance from kernels of sweet corn that had high cell division-promoting capacity in callus cultures. The material was a N⁶-substituted adenine [6-(4-hydroxy-3-methyl-*trans*-2-enylamino) purine] and was given the name **zeatin**, since it was isolated from maize (*Zea mays*).

Since the discovery of zeatin, several naturally occurring cytokinins and some synthetic substances with similar biological activities have been discovered. These substances, while they all carry an adenine moiety, differ in the structures of their side chains. Thus, like auxins, cytokinins are defined more on the basis of their biological functions than their structure.

2. BIOLOGICAL FUNCTIONS AND BIOASSAYS

Cytokinins are defined as compounds that promote cell division in callus and tissue culture. In combination with auxins, they regulate the ratio of shoot bud vs root growth in tissue culture and in stem cuttings; in intact plants, they regulate apical dominance and lateral root initiation. They also retard senescence and chlorophyll degradation in aging leaf tissues and, in combination with ethylene and light, regulate the growth of dicot seedlings in dark. These responses are covered in Sections III and V of this book.

Bioassays for cytokinin activity include (1) promotion of growth in tobacco pith culture or soybean callus tissue and (2) expansion of excised radish cotyledons in culture. These assays are based on the promotion of cell division and expansion activity by cytokinins. Another common bioassay is inhibition of senescence, as measured by a reduction in loss of chlorophyll from leaf tissues.

Cytokinins, like auxins and gibberellins, have a wide occurrence in plants and are known besides vascular plants from mosses and algae. In mosses they regulate bud growth in protonema. They are also produced by several strains of soil-living and phytopathogenic bacteria, such as *Agrobacterium* and *Pseudomonas*, which cause galls or tumors in plants, and by certain phytopathogenic fungi, such as *Helminthosporium* and *Ustilago* (see Appendix 2). Interestingly, an example of the inhibition of senescence by cytokinins is provided

by these fungi: they cause the formation of "green islands" on leaves of plants, which they infect.

3. STRUCTURE OF CYTOKININS

All naturally occurring cytokinins have an adenine ring structure with a 5 carbon isopentenyl side chain from N⁶ of the adenine molecule (Fig. 8-2). Some authors refer to these as the isoprenoid class of cytokinins. In addition to zeatin, they include **isopentenyladenine** (or dimethylallyl adenine) and the reduced form of zeatin, the **dihydrozeatin**. Because the side chain in zeatin possesses a double bond, geometric isomers are possible. These occur either as **trans-zeatin** or **cis-zeatin**. In the *cis* isomer, the hydroxyl group of the isopentenyl side chain is oriented toward the N¹ position of the purine ring, whereas in the *trans* isomer the hydroxyl group is oriented away from the purine ring.

Adenine occurs in DNA and RNA, and its ribose and ribose plus phosphate derivatives, adenosine and adenosine 5'-monophosphate, respectively, are common in plants. The ribose is always attached to N⁹ in the purine ring. Thus, natural cytokinins also occur as their sugar derivatives, N⁹ **ribosides** and **ribotides** (sugar plus phosphate). Figure 8-3 shows the structures for zeatin riboside and zeatin ribotide; others have similar structures.

In addition to ribosides and ribotides, conjugates of cytokinins with glucose, xylose, and amino acids are also known. Some authors treat all of these compounds together as natural cytokinins. Thus, there can be a bewildering array of naturally occurring cytokinins or cytokinin-type compounds. In this text, the term cytokinin is used, as far as possible, for natural cytokinins with an adenine base. Ribosyl derivatives are referred to as ribosylated derivatives, and conjugates are referred to as conjugates.

4. CYTOKININS OCCUR FREE IN THE CYTOPLASM AS WELL AS COMPONENTS OF tRNA

Natural cytokinins and their ribosides and ribotides occur free in the cytoplasm, but they also occur as integral components of certain transfer RNAs (tRNAs). As components of these tRNAs, they are always in the form of ribosylated derivatives, not as adenine bases. Many such ribosylated cytokinins are known from tRNAs (e.g., isopentenyladenine-9-riboside, *cis*- and *trans*-zeatin-9-riboside, 2-methylthioisopentenyl-

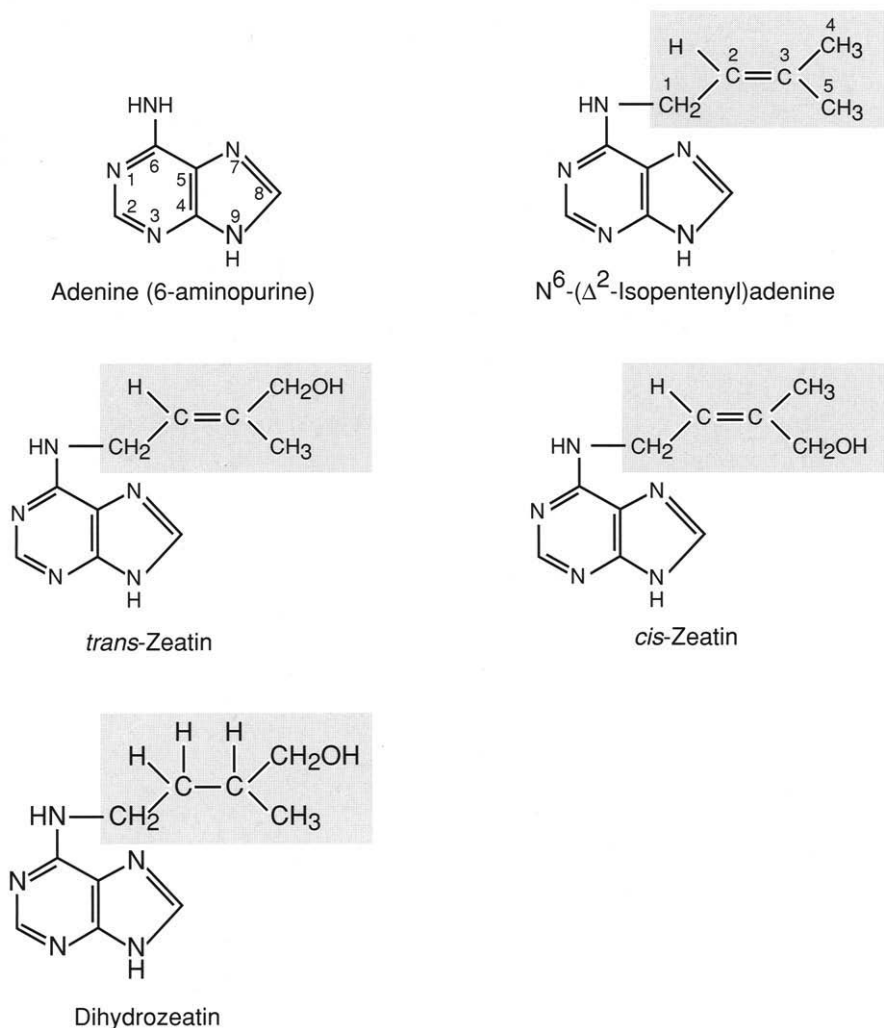


FIGURE 8-2 Structure of the isoprenoid class of cytokinins. (Top left) Adenine (6-aminopurine) with the ring numbering system. Natural cytokinins are all derivatives of adenine, with substitution of a five carbon chain in the amino group (shaded area). Carbon atoms in the side chain are numbered 1–5. Hydroxylation of the side chain at C-4 creates asymmetry and gives rise to *trans*- or *cis*-zeatin.

adenine-9-riboside, *cis*- and *trans*-2 methylthiozeatin-9-riboside). Such tRNA-bound ribosylated cytokinins occur widely in a variety of organisms: plants, phytopathogenic bacteria, yeast, and animals. Transfer RNAs are rich in substitutions (e.g., methylations), which occur all over the molecule, but the cytokinin ribosides occupy a precise location—they occur exclusively as the base adjacent to the 3' end of the anticodon (Fig. 8-4).

The function of such exclusive localization is obscure, but it has been suggested that it may play a regulatory role in protein synthesis, probably by promoting the codon–anticodon interaction and/or increasing the binding affinity of aminoacyl tRNA to the ribosomes. However, there is no direct proof for this assumption. The supply of exogenous cyto-

kinins to plant tissues is known to promote protein synthesis, but it is considered doubtful that such promotion is mediated *via* cytokinin derivatives bound to tRNA.

5. RELATIVE DISTRIBUTION OF NATURAL CYTOKININS AMONG PLANTS

It is likely that different plants show lesser or greater abundance of one or the other types of natural cytokinins. Among the plants that have been investigated, *trans*-zeatin (unless necessary, hereafter referred to as zeatin only) seems to be the most widely distributed.

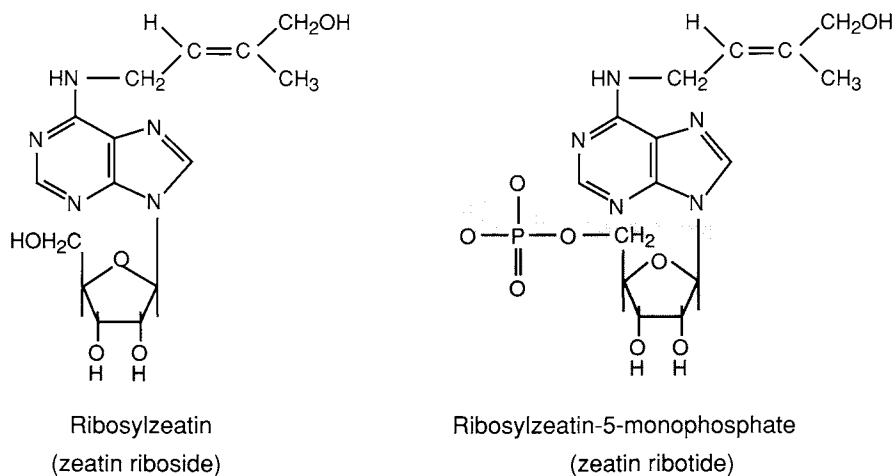


FIGURE 8-3 Structures of zeatin riboside and zeatin ribotide (zeatin is shown in *trans* configuration).

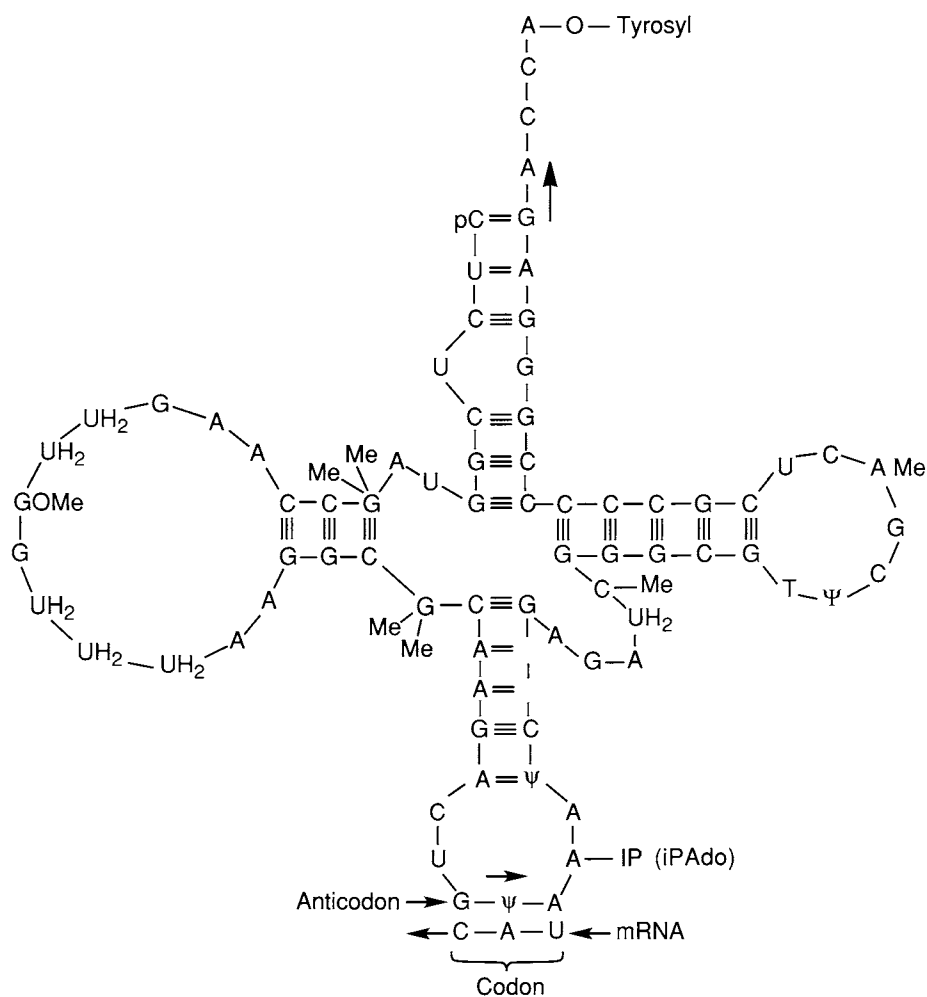


FIGURE 8-4 The structure of yeast tRNA for tyrosine. Note that the isopentenyladenosine (iPAdo) is the base next to the 3' end of the anticodon. From Hall (1970).

Zeatin is also one of the most biologically active cytokinins known. In contrast, *cis*-zeatin has much less activity. Until recently, *cis*-zeatin was thought to be present mainly in tRNA as a ribosylated derivative, and there were only sporadic reports of its occurrence as a free cytokinin. Studies using GC-MS for identification reveal, however, that *cis*-zeatin, as well as its ribosides and ribotides, in free form are the predominant cytokinins in chick pea seeds, whereas *trans* isomers are minor constituents. As more plants are investigated, it may turn out that both forms of zeatin occur in free form and that their relative abundance varies in different plants, or plant parts. Dihydrozeatin and its ribosyl derivatives and conjugates also occur frequently, but isopentenyladenine seems to have much less free occurrence.

6. BIOSYNTHESIS IN HIGHER PLANTS

6.1. The Postulated Pathway for Free Cytokinins

In Chapter 7, we noted that isopentenyl diphosphate (IPP) is the starting point for several plant hormones, gibberellins, brassinosteroids, and, *via* carotenoids, abscisic acid (see Fig. 7-5 in Chapter 7). It is also the starting point for the synthesis of the isoprenoid class of cytokinins. IPP isomerizes with dimethylallyl diphosphate (DMAPP or Δ^2 isopentenyl diphosphate). DMAPP, in turn, is believed to condense with adenosine-5'-monophosphate (AMP) to give rise to isopentenyladenosine-5'-monophosphate (iPMP) (Fig. 8-5).

iPMP is the precursor for all naturally occurring cytokinins. It is stereospecifically hydroxylated at C-4 of the side chain to form zeatin ribotide (ZMP). In a subsequent step, the ribose plus phosphate are cleaved to give zeatin. The hydroxylation step seems to occur very quickly, because iPMP, isopentenyladenosine (iPA), and isopentenyladenine (iP) are rarely found as free compounds in most plants. Zeatin, once formed, is stable and may be reduced to dihydrozeatin at a later step.

The scheme just given is based on feeding ^{14}C -labeled adenine to plant tissues or extracts, and analysis of products. It is difficult to be certain of the pathway because the products occur in low abundance and their radioactivity has to be measured against a

background of radioactivity in precursors that occur in large abundance, a situation very similar to that for IAA (see Chapter 6). Moreover, there are no known cytokinin synthesis mutants or inhibitors of cytokinin biosynthesis, and the enzymes associated with these reactions have not been completely purified (but see below).

6.2. Synthetic Enzymes

Several phytopathogenic bacteria that cause tumors in plants synthesize cytokinins via transfer of DMAPP to N⁶ of 5' AMP, and the bacterial genes encoding these isopentenyl transferases, have been cloned and characterized (see Appendix 2). Thus, it is likely that the first step in cytokinin biosynthesis in higher plants is catalyzed by a similar protein. However, efforts to purify the key enzyme, an isopentenyl transferase, also known as cytokinin synthase, have proven difficult, partly because it seems to be highly unstable, and partly because the substrates IPP/DMAPP and AMP, as well as the reaction product iPMP are targets for attack by phosphatases in plant tissues. In this dilemma, molecular techniques have come to rescue. An analysis of *Arabidopsis* genome has revealed nine gene sequences that encode putative isopentenyl transferases. Eight of these sequences expressed in *E. coli* yield recombinant proteins that show isopentenyl transferase activity. Among these, one gene, *AtIPT4*, gives a recombinant protein which catalyzes *in vitro* the transfer of the isopentenyl moiety from DMAPP to ATP with K_m values comparable to those for similar enzymes in bacteria. The recombinant *AtIPT4* can utilize ADP as well, but significantly does not utilize AMP, as a substrate. The enzyme is biologically significant because an overexpression of the sequence in transgenic *Arabidopsis* induces callus cultures to show cytokinin responses constitutively, that is, form shoots in the absence of added cytokinins. Thus, it seems that a major enzyme catalyzing the key step in cytokinin biosynthesis is a DMAPP:ATP/ADP isopentenyl transferase, rather than a DMAPP:AMP isopentenyl transferase.

Another gene sequence, *AtIPT1*, encodes a similar enzyme, which utilizes AMP but with a much higher K_m value. Based on these findings, a model for cytokinin synthesis in plants has been proposed (Fig. 8-5). The model postulates utilization of ATP, ADP as well as AMP, as substrates together with DMAPP. The

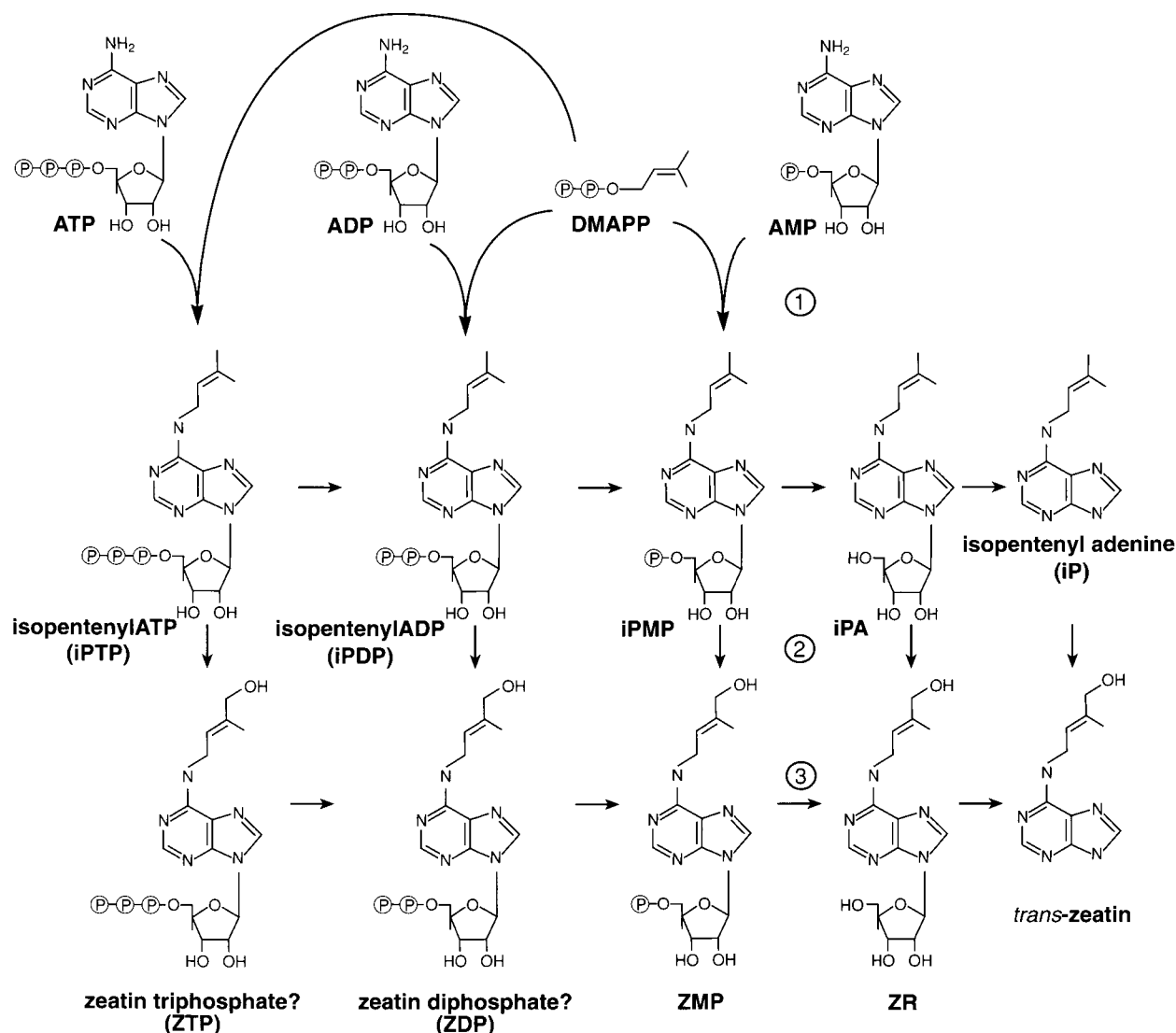


FIGURE 8-5 A model for the cytokinin biosynthetic pathway in plants. The model visualizes the transfer of the isopentenyl moiety of DMAPP to ATP, ADP, or AMP by isopentenyl transferases (step 1) to give rise to corresponding isopentenyladenosine-tri-, di- or monophosphates (iPTP, iPDP, or iPMP). The tri- or diphosphates are thought to funnel into the iPMP. In step (2), iPMP is hydroxylated at C-4 in the side chain to give rise to zeatin monophosphate (ZMP). Similar hydroxylations can also occur in iPTP or iPDP to yield corresponding zeatin tri- or diphosphates (ZTP or ZDP), which on subsequent dephosphorylations can give rise to ZMP. In step (3), ZMP is converted to zeatin riboside which is then cleaved to give rise to zeatin and sugar, or cleaved directly to give zeatin and sugar phosphate. Similarly iPMP gives rise to iPA and iP, which can also be hydroxylated to ZR and zeatin, respectively. The products that are conjectural are indicated by question marks. From Kakimoto (2001).

precise steps and reaction products are still unknown, but the pathway is thought to progress via iPMP to ZMP to zeatin.

The hydroxylation at C-4 of the side chain of iP and iPA and iP to ZR and zeatin, respectively, is catalyzed by a membrane-bound cytochrome P450 monooxygenase. It is likely that the same enzyme also catalyzes the conversion of iPMP to ZMP in step 2 of

the synthesis of zeatin (see Fig. 8-5). The conversion of ZMP to ZR and zeatin and similar conversions of iPMP to iPA and iP in step 3 (Fig. 8-5) are probably accomplished by generic enzymes catalyzing the conversion of nucleotides to nucleosides and nitrogen bases.

An alternate view has also been advanced that in *Arabidopsis* zeatin-type cytokinins are produced inde-

pendently of iPMP. In the model shown in Fig. 8-5, if one of the methyl groups in iPTP or iPDP are hydroxylated to produce zeatin-ribosyl-5'-tri- or diphosphate (ZTP or ZDP) followed by dephosphorylation, an iPMP-independent origin of zeatin can occur.

6.3. Formation of Cytokinins from tRNA

Because tRNAs of plants contain ribosylated cytokinins (see Section 4 and Fig. 8-4), there has been a persistent theme for a long time that free cytokinins arise from the degradation of tRNAs. While it is possible that small amounts of free cytokinins arise in this manner, the consensus is that this is not the major route for production of free cytokinins. There are several reasons. Some are outlined below:

i. In the tRNA of plants and plant-associated bacteria, the *cis* isomer of zeatin generally predominates over the *trans* isomer by a ratio of almost 40:1. The ratios of the two isomers in the free form in cytoplasm are the reverse, where *trans*-zeatin predominates and the *cis* form is scarce.

ii. Certain tissue culture lines of tobacco require an exogenous supply of cytokinins for growth, whereas other lines are cytokinin autonomous. In lines that are cytokinin dependent, ribosylated derivatives of cytokinins still occur in the tRNAs. If the tRNAs were a major source of free cytokinins, cytokinin dependence of such cultures is hard to explain. Also, in lines that are cytokinin autonomous, the tRNA pool does not seem to turn over at a high enough rate to account for the pool of free cytokinins in the cytoplasm.

As mentioned earlier, *cis*-zeatin and its ribosides/ribotides have been shown to be the predominant form of free cytokinins in chick pea seeds. The synthesis of these *cis* forms is still a mystery.

In summary, there is little support for the origin of free cytokinins from degradation of tRNAs. Instead, it has been thought that the synthesis of cytokinins occurs *via* transfer of DMAPP to AMP in a manner analogous to that seen in many phytopathogenic bacteria. This view has been challenged recently by the isolation of gene sequences in *Arabidopsis* that encode isopentenyl transferases that can catalyze *in vitro* the transfer of DMAPP to ATP/ADP or to a lesser extent AMP. Moreover, an overexpression of the gene for DMAPP:ATP/ADP isopentenyl transferase in transgenic tissue gives cytokinin responses constitutively. Thus, plants seem to utilize a novel type of isopentenyl transferase that has greater affinity for ATP/ADP than AMP. Since many gene sequences in *Arabidopsis* encode isopentenyl transferases, it also seems

likely that multiple isoforms of the enzyme occur in plants. The details of reaction products and steps involved in biosynthesis of zeatin starting from ATP/ADP and DMAPP are still unknown, but are thought to progress via iPMP. There is also evidence that zeatin may be formed independently of the iPMP pathway.

6.4. Sites of Synthesis

Cytokinins are believed to be synthesized in young or meristematic tissues, e.g., root apices, developing shoot buds, cambial tissue, developing seeds, especially liquid endosperm, and young fruits; in short, in areas where cell divisions are occurring at a high frequency. The cloning of the isopentenyl transferase genes is likely to provide definitive information as to the sites of cytokinin synthesis and environmental factors that regulate such synthesis. The cytokinins produced in roots tips are known to migrate upward through the xylem, as can be shown by collecting xylem sap from cut stem stumps and analyzing the sap. Likewise, phloem exudate coming from apical buds can be collected and shown to contain cytokinins (see Chapter 13).

There is not much reliable information on endogenous levels because very few plants/parts have been investigated using GC, GC-MS. From bioassays, levels of 1–100 $\mu\text{g} \cdot \text{g fw}^{-1}$ are reported, lesser in vegetative parts, and more in seeds and storage tissues, but in these latter tissues, they occur mostly in conjugated forms (see Section 7.2).

7. REGULATION OF CYTOKININ LEVELS

As in the case of IAA and GAs, regulation of the free cytokinin content is critical for orderly plant growth and development. This is shown clearly by plants that overproduce cytokinins either because of a mutation or because they have been transformed by the *ipt* gene from *Agrobacterium*.

As mentioned earlier, mutants in cytokinin biosynthesis have not been identified, but a cytokinin overproducing mutant, *amp1* (for altered meristem program), is known from *Arabidopsis*. This mutant was isolated in a screen for altered morphologies in seedling growth and proved to contain five to six times higher concentrations of cytokinins. The mutant plants show several abnormalities, including production of multiple cotyledons in the embryo, enhanced lateral branching, retarded root development, and delayed

senescence of leaves. Except for polycotyly, similar effects are seen in plants transformed with and overexpressing the bacterial *ipt* gene. These cytokinin-overproducing plants also show enhanced levels of cytokinin metabolites.

7.1. Regulation of Cytokinin Biosynthesis

Factors that trigger or modulate the synthesis of cytokinins are still mostly unknown, but with the cloning of the *IPT* genes in *Arabidopsis*, rapid progress is expected.

However, there is considerable information on cytokinin metabolism. This information comes mainly from feeding labeled zeatin or dihydrozeatin to plant tissues or organs and analysis of labeled products. Because cytokinins are known to be synthesized in roots and translocated to aboveground parts, one common method of feeding exogenous cytokinins is *via* cut ends of seedlings with roots removed. Other favorite materials for feeding experiments are immature seeds, fruits such as kernels or pods, or cut leaves.

7.2. Conjugation of Cytokinins

7.2.1. Formation of Ribosyl Derivatives

Tritiated zeatin and dihydrozeatin fed to plant tissues are rapidly converted to their N⁹-ribosides and ribotides as shown in Fig. 8-6 (for structures of these molecules, see Fig. 8-3). Free cytokinins and their ribosides, and ribotides are interconvertible. In a two-step process, zeatin can be converted to zeatin riboside by adenosine phosphorylase, and zeatin riboside can be converted to the ribotide form by an adenosine kinase. Zeatin can also be directly converted to its ribotide by an adenosine phosphoribosyltransferase (APRT). Enzymes involved in these interconversions are generic enzymes, which catalyze adenine to

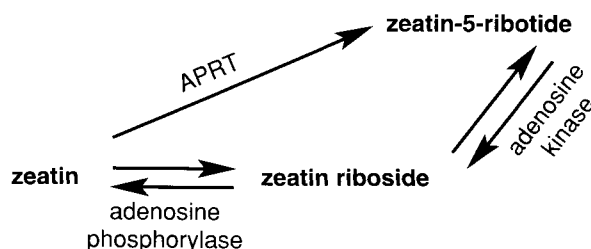


FIGURE 8-6 Schematic illustration of interrelationships among zeatin, zeatin riboside, and zeatin ribotide. APRT, adenosine phosphoribosyl transferase.

nucleotide conversions. For example, an APRT, isolated and characterized from *A. thaliana*, can catalyze phosphoribosylation of not only free cytokinin bases, such as zeatin, but also adenine as a substrate. The ribosides and ribotides show biological activity in bioassays, but most likely they are not active *per se* and it is the free N bases released after hydrolysis that are active.

As mentioned in Section 4, zeatin ribotide is also formed during the synthesis of zeatin (see Fig. 8-5) and can give rise to zeatin riboside. Thus, ribosides and ribotides are intermediates in the biosynthesis of cytokinins and also represent forms that scavenge excess active cytokinins. The regulation of these pools of cytokinins and their ribosyl derivatives is little understood but likely involves different enzymes in the two sets of reactions and probably different cellular compartments. There also seems to be a homeostatic control over their levels. For instance, after continuous feeding of cytokinins or ribosyl derivatives to plant tissues, excess ribosides and ribotides may be irreversibly converted to N-glucosylated derivatives or be broken down by side chain cleavage (see Section 7.3).

7.2.2. Glycosylation of the Side Chain

Sugars such as glucose or xylose can be conjugated *via* the -OH group in the side chain forming what are known as O-glycosides (Fig. 8-7). These reactions are reversible. O-glycosides, like ribosides and ribotides, show activity in bioassays, but it is generally accepted that free bases provide the activity.

O-glycosides are very common and seem to be the principal form in which the cytokinins are stored in storage tissues and developing seeds, where they may accumulate to surprisingly high levels. Their synthesis involves specific glycosyl transferases, and genes encoding a zeatin-O-glucosyl transferase (*ZOG1*) and a zeatin-O-xylosyl transferase (*ZOX1*) have been cloned from *Phaseolus* species. With cloning of these genes, more information about the sites of glycosyl conjugation and factors that regulate it is likely to accumulate. Because of their increased polarity, O-glycosylated conjugates can be sequestered into vacuoles for possible use later.

The hydrolysis of O-glycosyl conjugates occurs readily, and several β -glycosidases, are known to cleave the O-glycosyl group. In the absence of β -glycosidases, O-glycosides are relatively stable in plant tissues. They are protected against oxidative cleavage of the side chain by the main degradative enzyme cytokinin oxidase (see later) because of the presence of the glycosyl residue in the side chain.

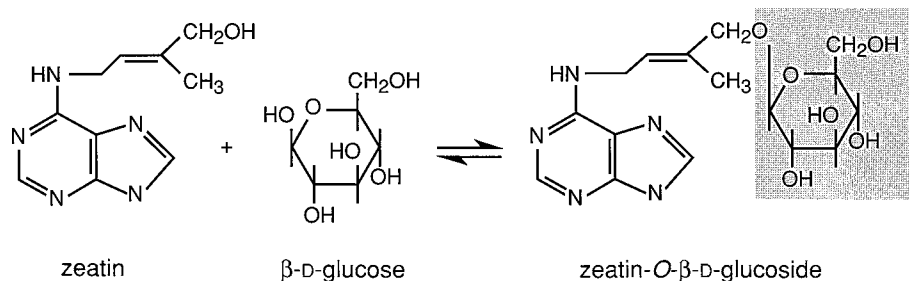


FIGURE 8-7 Glycosylation of the side chain in zeatin.

7.3. Irreversible Inactivation

Cytokinins are irreversibly inactivated by certain modifications in the purine ring structure or by cleavage of the N⁶ side chain.

7.3.1. Conjugation of Glucose or Amino Acid Residues with Adenine

Glucose conjugates at the N⁷ or N⁹ position in the adenine ring (e.g., [7Glc]Z or [9Glc]diHZ) are formed readily if zeatin or dihydrozeatin is supplied to plant tissues (Fig. 8-8A). N³-glucosides are also formed but appear to be less common. Such position-specific glucosylations are probably catalyzed by specific glucosyltransferases, not the ones involved in O-glycosylations, but the details are obscure.

Amino acid conjugates with alanine at the N⁹ position are also formed (Fig. 8-8B). They were first isolated as minor metabolites in lupin (*Lupinus luteus*) and, hence, were named lupinic acid (conjugate with zeatin ([9Ala]Z) and dihydrolupinic acid (conjugate with dihydrozeatin (diH[9Ala]Z)). An enzyme, a β-(6-allylaminopurine-9-yl) adenine syn-

thase, has been characterized from developing seeds of lupin.

N-glucosylated or amino acid conjugates are stable over long periods and seem to be irreversibly inactivated products. Enzymes capable of hydrolyzing these products have not been investigated. These compounds are much more polar than the original cytokinins, which may facilitate their sequestration in the vacuole.

7.3.2. Oxidative Cleavage of the N⁶ Side Chain

Cytokinin oxidase is the enzyme that catalyzes the cleavage of the N⁶ side chain from adenine. The enzyme requires the Δ² double bond in the side chain for its activity. Hence, its natural substrates are isopentenyladenine and zeatin and their ribosylated derivatives (Fig. 8-9). The enzyme is unable to cleave side chains that lack the double bond, as in dihydrozeatin and its derivatives. It is also inactive against O-glycosylated conjugates, where the side chains have a glucosyl or xylosyl residue (see Fig. 8-7), and against synthetic cytokinins (e.g., benzyladenine, kinetin), where the side chains have an aromatic ring (see later).

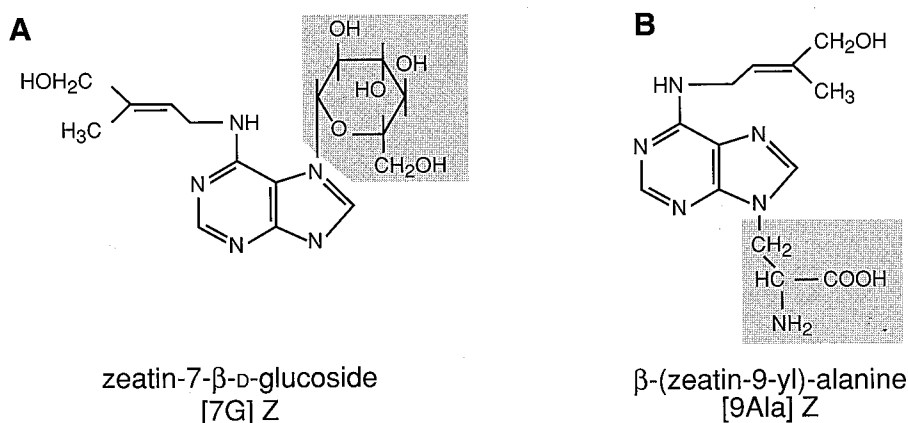


FIGURE 8-8 N-conjugation of the adenine ring by glucose (A) or alanine (B).

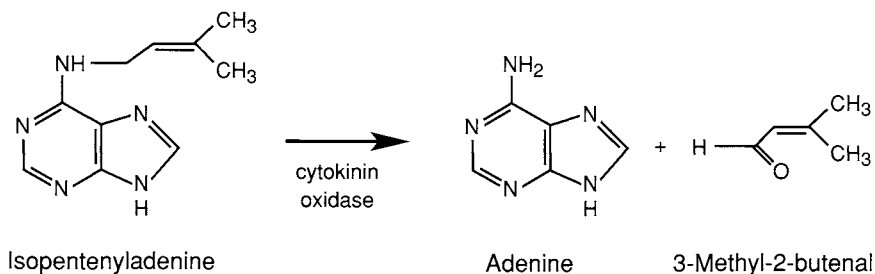


FIGURE 8-9 Cleavage of the side chain of isopentenyladenine by cytokinin oxidase. The products from isopentenyladenine are adenine and 3-methyl-2-butenal.

8.3.2.1. Cytokinin Oxidase Activity Is Enhanced by Its Substrate

As mentioned before, the endogenous levels of free cytokinins are strictly regulated. Cytokinin oxidase activity is increased severalfold if plant tissues are incubated with large quantities of cytokinins *in vivo*. Similarly, an increase in cytokinin oxidase activity is seen in tobacco plants transformed with the *ipt* gene from *Agrobacterium*. These data indicate that cytokinin oxidase activity is induced by its own substrate and provide an elegant example of a negative feedback control over the endogenous levels of bioactive hormones.

8.3.2.2. Cytokinin Oxidase Activity Varies in Different Tissues and Is Developmentally Regulated

The N-glucosylation of the purine ring and side chain cleavage are two methods for irreversibly lowering the intracellular levels of biologically active cytokinins, but the degree to which they are practiced in different plants, or plant parts, seems to vary. For instance, in radish seedlings, cytokinin oxidase activity is minimal, and more N⁷- than N⁹-glucosylation is favored. In corn seedlings and embryos in culture, N⁹-glucosides are more common.

Cytokinins are abundant in embryo and endosperm and in fruit pericarp during early phases of seed and fruit development (see Chapters 17 and 18). These levels are gradually reduced and there is a progressive increase in cytokinin oxidase activity in tissues as cell divisions cease and tissues mature. For instance, in developing fruits (pods) of *Phaseolus* sp., the degradation of zeatin by cytokinin oxidase was more rapid in seed coat tissue (where cell divisions cease earlier) than in the embryo or fruit tissue. In corn kernels, cytokinin oxidase activity increased as maturation progressed.

8.3.3.3. The Gene for Cytokinin Oxidase Has Been Cloned

CK oxidase was purified to homogeneity from immature maize kernels, and the sequence used to clone

its gene called *CKX1* (for cytokinin oxidase1, the authors use the designation *ckx1*). The enzyme is an oxidoreductase and requires flavin adenine dinucleotide (FAD) and molecular O₂ for activity. It can use *trans*-zeatin, isopentenyladenine, and isopentenyladenosine, as substrates, but not, as expected, dihydrozeatin. Several genes from *Arabidopsis* have been cloned also, and detailed studies on the enzyme kinetics are in progress using recombinant protein.

The cloning of cytokinin oxidase genes in maize and *Arabidopsis* and O-glycosylating enzymes in *Phaseolus* spp are important first steps in our understanding of cytokinin metabolism in plants. More work is likely to follow, and show whether these genes are regulated at the transcriptional or translational level, whether there are different isoforms with tissue and/or substrate specificities, and what specific factors cause their induction.

8. SYNTHETIC COMPOUNDS WITH CYTOKININ-LIKE ACTIVITY

Kinetin was originally obtained from herring sperm DNA, and its discovery led to a search for synthetic compounds that could mimic cytokinin activity. Several compounds were isolated that had high activity in promoting cell division in tobacco pith culture. One of the most potent compounds isolated was 6-benzylaminopurine or benzyladenine (BA) (Fig. 8-10). Like all other cytokinins, BA has an adenine ring with an N⁶ side chain, but the side chain carries an aromatic ring, as in kinetin. Other synthetic compounds include several hydroxylated benzyl derivatives (Fig. 8-10).

Benzyladenine and kinetin are two of the most widely used cytokinins. Benzyladenine has the added advantage that it is easily available and is relatively inexpensive. Both are stable compounds because, unlike zeatin and isopentenyladenine, their

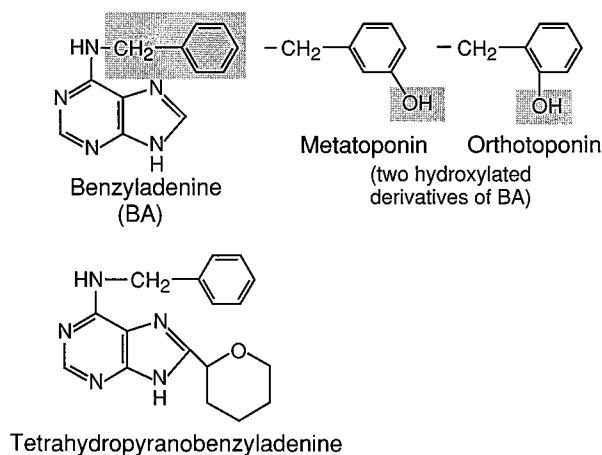


FIGURE 8-10 Structure of benzyladenine and some other synthetic cytokinins.

side chains are immune to attack by cytokinin oxidase. The glucosylated conjugates of the side chain are also not formed because of the differences in the side chain. However, the enzymes that catalyze the modifications of the purine ring, i.e., formation of ribosylated derivatives and N-substituted glucosides and amino acid conjugates, are able to use BA and kinetin as substrates. Thus, BA administered exogenously is known to give rise to its N⁹-ribosyl derivatives, as well as N³-glucoside and [9Ala] conjugates.

Some authors refer to these cytokinins as aromatic cytokinins. Although aromatic cytokinins are still considered as of nonplant origin, reports suggest that they occur naturally in plants. Thus, benzyladenine and its hydroxylated derivatives have been reported in several plant tissues on the basis of immunoassays. These reports need to be confirmed by GC-MS analysis. Kinetin has also been reported to occur naturally and be formed *via* oxidative degradation of DNA *in vivo*.

8.1 Phenylurea Derivatives

Some other substances, such as modified diphenyl and pyridyl-phenyl urea derivatives (Fig. 8-11), display significant cytokinin activity in several bioassays. They stimulate cell division in tobacco pith culture, promote shoot formation in callus tissue, and retard leaf senescence; however even those derivatives that have the highest activity in cell division assay still are only 1/100th as active as the adenine cytokinins, such as benzyladenine. Their mode of action is not known, but they probably function by inhibiting endogenous cytokinin oxidase, thereby having a “sparing” effect on cytokinins present in the tissue by inhibiting their breakdown.

9. CYTOKININ ANTAGONISTS (ANTICYTOKININS)

Several synthetic compounds are known that show high anticytokinin activity. The most potent and specific of these appear to be substituted pyrrolo [2,3-*d*]-pyrimidines and 7-substituted-3-methylpyrazolo [4,3-*d*]pyrimidines (Fig. 8-12). At low doses (~ 1 μM), these molecules specifically inhibit cytokinin-induced growth in cytokinin-dependent tobacco tissue cultures. Although their mode of action is not known, they seem to act as competitive inhibitors because

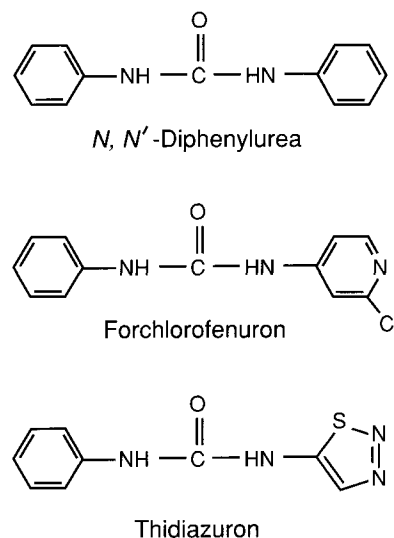


FIGURE 8-11 Some phenylurea derivatives with cytokinin activity: *N,N'*-Diphenylurea; *N*-(2-chloro-4-pyridyl)-*N'*-phenylurea (trade name, Forchlorfenuron); and *N*-phenyl-*N'*-(1,2,3-thiadiazol-5-yl)urea (Thidiazuron). From Shudo (1994).

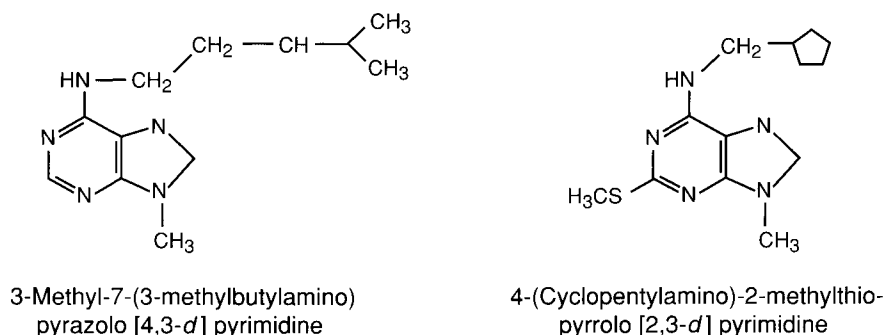


FIGURE 8-12 Two potent anticytokinins: 3-methyl-7-(3-methylbutylamino) pyrazolo [4,3-*d*]pyrimidine and 4-(cyclopentylamino)-2-methylthiopyrrolo[2,3-*d*]pyrimidine.

inhibition can be reversed by increasing the cytokinin concentration in the medium.

10. CHAPTER SUMMARY

Cytokinins serve many important functions in plant development and morphogenesis. They are involved in the regulation of cell division; they interact with auxins in the control of apical dominance and lateral branching and the root–shoot ratio in intact plants and in tissue culture. They retard the senescence of leaves and promote the light-independent deetiolation response, including greening, of dark-grown seedlings. Several cytokinins occur naturally in plants. They have an adenine base and a five carbon isopentenyl side chain. Among these, zeatin, specifically *trans*-zeatin, is the most abundant. The synthesis of cytokinins in higher plants has been unclear and controversial for a long time, but progress finally seems to be achieved with the cloning of genes encoding isopentenyl transferases (IPTs) in *Arabidopsis*. These IPTs seem to use ATP/ADP, rather than AMP, together with DMAPP, to yield isopentenyladenine monophosphate (iPMP), which ultimately gives rise to zeatin and other naturally occurring adenine cytokinins. The possibility that cytokinins may arise from the degradation of tRNAs is not considered likely. Ribosylated derivatives, with a ribose or ribose 5'-monophosphate attached to the adenine moiety, of cytokinins are common. Conjugates of cytokinins where a glycosyl moiety is attached to the -OH group in the side chain are also common. Both ribosylated derivatives and glycosyl conjugates show activity in bioassays, but it is generally believed that they do so after hydrolysis to free bases. Irreversible deactivation of cytokinins occurs in two ways. The side chain may be cleaved by cytokinin oxidase, an enzyme that has specific requirements for an -OH

group and a $\Delta 2,3$ bond in the side chain. Significantly, this enzyme is induced by high concentrations of cytokinin in plant tissues. Modifications to the adenine ring, by substitution of glucose or amino acids (particularly alanine) to the nitrogen atoms, also cause irreversible deactivation. Genes encoding cytokinin oxidase and glycosylating enzymes have been cloned from several plants and rapid progress in our understanding of regulation of cytokinin metabolism is expected. Several synthetic cytokinins (e.g., benzyladenine, kinetin) are known. They have the adenine base, but have an aromatic ring in the side chain. Supplied to plant tissues, these cytokinins form ribosylated derivatives and glucosyl or amino acid substituents in the adenine ring, but they are more stable (than zeatin) in plant tissues because they are not subject to side chain cleavage by cytokinin oxidase. There are some reports that benzyladenine and kinetin occur naturally. Some synthetic compounds, which inhibit cytokinin promotion of cell division in tissue/cell culture, probably as competitive inhibitors, are known.

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Brassinosteroids

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1. DISCOVERY

Plant pollen has been considered for a long time to be rich in compounds with growth-promoting activities. In 1970, an oily extract from pollen of rape (*Brassica napus*) was shown to cause extreme elongation of the internodes of pinto bean (Mitchell *et al.*, 1970). Following this discovery, a major screening effort was mounted by the U.S. Department of Agriculture. Pollen extracts from some 60 different species were tested in various bioassays and shown to have varying amounts of growth-promoting activities, with pollen from rape and alder (*Alnus glutinosa*) showing some of the highest activities.

The identity of the active compound in these extracts, known as "brassins" (so called because the first extracts were from the genus *Brassica*), was unclear. That they were not gibberellins (GAs) was indicated by some differences in growth pattern, such as swelling and curvature of stem, which do not occur in GA-induced growth. A large-scale extraction and purification program was undertaken. Some 227 kg of rape pollen was extracted and fractionated with activity tests at each step. Finally, 4 mg of crystals was obtained from 40 kg of pollen, and the active compound was identified as brassinolide (Grove *et al.*, 1979). Brassinolide [(22*R*,23*R*,24*S*)-2 α ,3 α ,22,23-tetrahydroxy-24-methyl- β -homo-7-oxa-5 α -cholestan-6-one] was the first plant steroid with hormonal activities (Fig. 9-1).

Since then, many other steroidal compounds with growth-promoting activities have been isolated from plants; some 40 are known. In this book, they are collectively referred to as brassinosteroids (BRs), but individual compounds are designated by their names.

2. STRUCTURE AND DISTRIBUTION

Brassinosteroids contain the typical steroid nucleus, with fused rings A, B, C, and D and an alkyl side chain at C-17. Both the nucleus and the side chain contain various substituents in different isomeric configurations, which make the stereochemistry and nomenclature of BRs highly complicated [for details, the reader is referred to the article by Mandava (1988)].

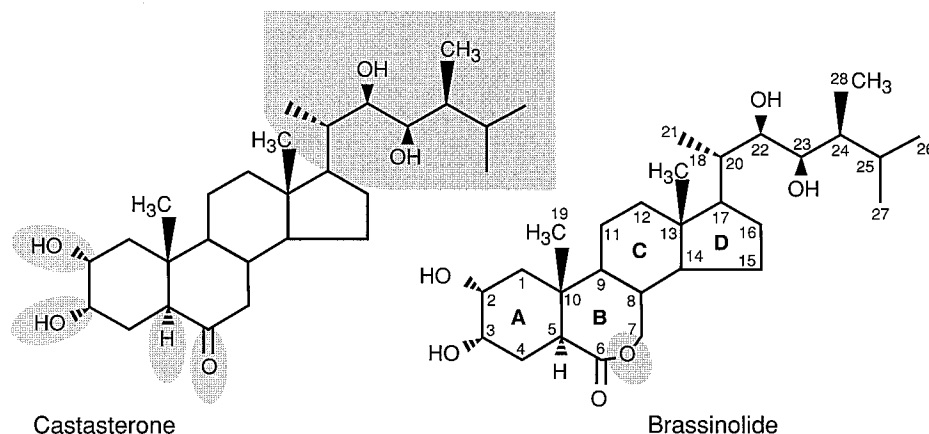


FIGURE 9-1 Structures of brassinolide and castasterone, the two most common BRs in plants. Parts of the molecules referred to in the text are shaded.

The two most common BRs in higher plants, the brassinolide (BL) and its ketone precursor, castasterone (CS), are C_{28} brassinosteroids (Fig. 11-1). Both have α -oriented hydroxyl groups (*cis* configuration) at C-2 and C-3 in ring A, an α orientation at C-5 (A/B ring junction), a ketone group at C-6 in ring B, β -oriented C-18 and C-19 methyl groups, α -oriented hydroxyl groups at C-22 and C-23 in the side chain, and an α -methyl at C-24. BL, in addition, has a 7-oxa-6-ketone (or 7-oxalactone), the first naturally occurring steroid with such a seven-membered lactone ring. Other naturally occurring BRs differ in the presence or absence of a ketone or a lactone in the B ring, in the number of hydroxyl groups in the A ring, and in the nature of substituents and their α vs β configuration in the side chain.

BRs are ubiquitous in their distribution in plants. So far, they have been found in dicots, monocots, gymnosperms, a fern, and an alga. They occur in almost all parts of the plant; they are most abundant in pollen (in rape pollen as much as $100 \text{ ng} \cdot \text{g fw}^{-1}$) and in immature seeds, but they are also present in young leaves, stems, and flower buds in smaller amounts (nanogram or subnanogram per g fresh weight). They probably occur in roots, although this needs confirmation. Some insect-induced galls, as well as crown galls induced by *Agrobacterium tumefaciens* (see Appendix 2), have relatively high levels of BRs.

3. PHYSIOLOGICAL ROLES AND BIOASSAYS

The most well-documented role of BRs is in promoting the elongation of stems, petioles, and flower

peduncles in dicots and of coleoptiles and mesocotyls in monocots. Exogenous application of BRs causes elongation in young-growing regions in both intact seedlings and cut segments (Fig. 9-2). Moreover, BRs are essential for stem elongation. Genetic mutants that are deficient in endogenous BRs, first characterized in 1996, are now known from *Arabidopsis*, tomato, and pea (see Section 5). These mutants are dwarfs compared to the wild type, whether grown in light or dark, and an exogenous application of BL (or CS) restores them to the wild phenotype.

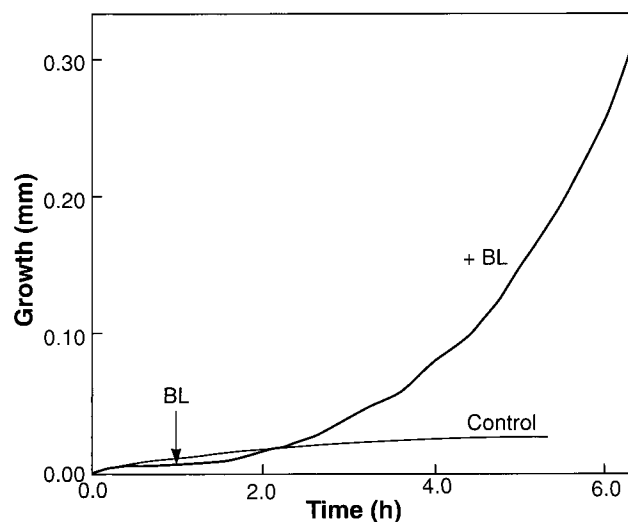


FIGURE 9-2 Stem elongation by exogenous brassinolide (BL) in soybean (*Glycine max*). Epicotyl segments treated with BL or without (control) were monitored continuously for elongation growth using a special instrument and a recording device (for this method, see Chapter 15). Acceleration of growth began 45 min after addition of BL and reached a maximum steady state after 4–5 h. From Zurek *et al.* (1994).

Light is a strong regulator of plant growth. Gibberellins, cytokinins, and BRs interact with light in modulating such development. This topic is covered in Chapter 26, but it can be mentioned here that BR-deficient mutants, grown in the dark, show an unusual expression of features typical of seedlings grown in the light. For example, they lack an apical hook, have open expanded cotyledons, form the first leaves, and express genes related to photosynthesis and/or anthocyanin production. The phenomenon is seen in BR-deficient mutants of some plants (e.g., *Arabidopsis*, tomato), but not in others (e.g., pea)

The effect of BRs on cell division is not very clear. In BR-deficient mutants, exogenous BRs contribute to organ elongation by enhancing cell elongation, not by increasing the rate of cell division—the cell number in the organs examined is about the same as in the wild type. In some other systems, however, BRs are reported to induce cell division. Among other physiological roles, BRs inhibit root formation and root growth. Auxins also inhibit root growth, but they are essential for lateral and adventitious root initiation (see Chapter 14). Exogenous BRs are also reported to promote xylem differentiation, pollen tube growth, and male fertility in flower development. They also seem to promote ethylene production, influence senescence and abscission of plant organs, and enhance gravitropism. A recent report implicates them in the enhancement of seed germination in *Arabidopsis*. As more BR-deficient mutants are discovered and used for physiological studies, these roles are likely to be better elucidated.

There are many reports about the beneficial effects of exogenously applied BRs on yield, general vigor, and stress tolerance of agricultural and horticultural crops. Unfortunately, these reports so far have been inconsistent. Because BRs are structurally similar to ecdysteroids, the molting hormone of insects and other arthropods, and interfere with the binding of ecdysteroids to their receptor, it is believed that they offer protection against insect attack by acting as anti-ecdysteroids.

Two common bioassays for BRs include the bean second internode elongation assay and the rice leaf lamina inclination assay.

The bean internode elongation assay uses bean (*Phaseolus vulgaris* cv Pinto) seedlings when the second internode from the root-shoot junction is no more than 1 mm long, i.e., is still growing. GAs are active in this bioassay, but cause only elongation of the treated and upper (third and fourth) internodes. BRs characteristically evoke elongation accompanied by curvature and swelling and splitting (see Chapter 15). Such a response is sometimes referred to as the “brassin” response; its magnitude depends on the BR concentration. The

reason for splitting is unknown, but probably is due to an imbalance among water uptake, turgor pressure, and wall strength. Auxins are inactive in this bioassay.

The rice leaf lamina inclination assay, which is used extensively for the identification of BRs during a purification protocol and determining the potency of natural and synthetic BRs, utilizes the bending of the lamina (or blade) of rice leaves away from the sheath when exposed to BRs. Explants consisting of lamina, lamina joint, and leaf sheath from etiolated rice seedlings are floated on test solutions and bending of the leaf lamina is measured (Fig. 9-3). In a modification, intact seedlings of dwarf rice (*Oryza sativa* cv Tanginbozu and Waito-C) are used, and test solutions are applied as a microdrop at the junction between the lamina and the sheath. Auxins may give a positive test, but at much higher concentrations than those of BRs. Significantly, however, application of auxin (1–5 μg /assay), before, after, or with BR, has a synergistic effect in that sensitivity to BRs is enhanced 100- to 300-fold over that due to BRs alone. The reason for the bending is not clear and may involve an epinastic response due to the production of ethylene. GAs evoke a straight growth response without the bending characteristics of BRs.

4. BIOSYNTHESIS OF BRASSINOLIDE

Castasterone and brassinolide are the two most common and abundant BRs in higher plants; among the two, BL shows about five times higher activity in bioassays. Hence, efforts have been devoted to elucidating the biosynthetic pathway of BL. In this effort,

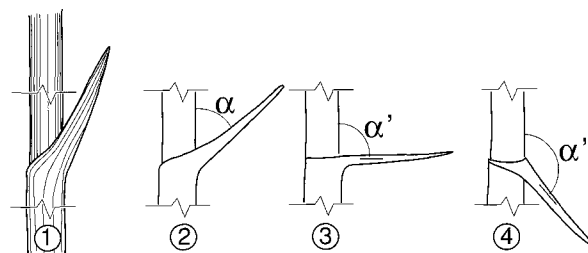


FIGURE 9-3 Rice leaf lamina inclination assay. Rice seedlings are grown in the dark for seven days, and the second leaf is excised below the lamina-sheath junction. The explant, comprising lamina, lamina joint, and leaf sheath, is floated in distilled water for 24 h before being incubated in the test solution in buffer for 48 h in the dark. The angle between the sheath and the lamina blade, which varies between rice varieties, increases severalfold in BR-treated material over control.

the availability of a cell suspension culture from *Catharanthus roseus* (the Madagascar periwinkle), which produces abundant quantities of BRs, and the feeding of deuterated presumed precursors were both very useful. Cell suspension cultures obviate the problems associated with uptake and transport of fed precursors in intact plants; they can be kept sterile and are readily harvestable for extraction and analysis. Elucidation of the biosynthetic pathway for BL in the last decade is the achievement of Shozo Fujioka and Akira Sakurai and their colleagues in Japan, and this pathway has been confirmed by the analysis of BR synthesis mutants. Brassinosteroids are synthesized from sterols, and our discussion begins with sterols and their roles in plants.

4.1. Sterols and Their Roles in Plants

A precise definition of sterols is difficult, but generally they are considered as triterpenoid (C_{30}), steroidal alcohols characterized by a 3β -hydroxyl group (chemically, they are triterpenes with a 3β -monohydroxy perhydro-1,2-cyclopentanthrene ring system).

They are derived from squalene, which, as shown in Chapter 7, is, in turn, derived from hydroxymethylglutaryl CoA (HMG-CoA) via mevalonic acid (MVA) (see Figs. 7-5 and 7-6). Squalene undergoes 2,3 epoxidation followed by cyclization to give rise to polycyclic triterpenoids (Fig. 9-4). It is generally accepted that the steps leading up to squalene 2,3-oxide from HMG-CoA are common in animals, fungi, and plants. After that the pathways diverge and yield a variety of cyclic triterpenoids (e.g., the pentacyclic β -amyrin, tetracyclic lanosterol, and cycloartenol), catalyzed by different cyclases. In nonphotosynthetic organisms, squalene 2,3-oxide is cyclized to lanosterol; whereas in photosynthetic organisms, it is cyclized to cycloartenol. Cycloartenol, after a series of modifications, gives rise to the large number of plant sterols. Some 300 different sterols are found in plants, among which sitosterol, stigmasterol, and campesterol are the most common. Cholesterol is also found, but is a minor component of plant sterols.

Sterols serve two major functions. They are important constituents of cellular membranes where they modulate membrane stability, possibly by forming esters with fatty acids and thus limiting their lateral mobility. They also modulate membrane permeability. Among the cellular membranes, the plasma membrane has the highest sterol content and sterol:phospholipid ratio. Thus, for cell division and growth, sterol synthesis is required, and, as expected, inhibitors of sterol synthesis retard growth. The other major function of sterols is to serve as precursors for steroid hormones in insects and mammals and for brassinosteroids in plants.

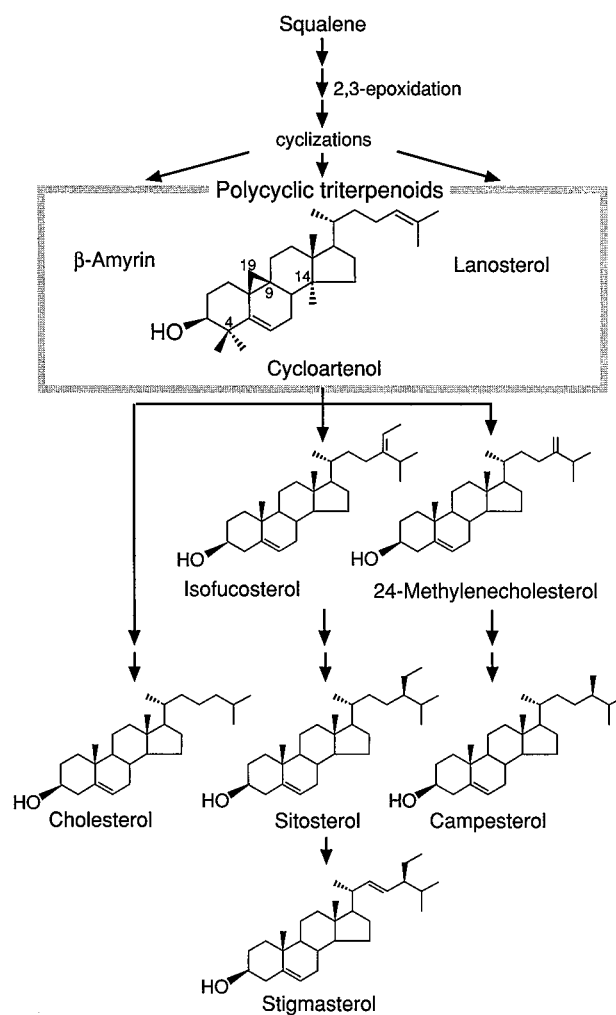


FIGURE 9-4 Major steps in sterol biosynthesis in plants. Squalene, derived from mevalonic acid, undergoes epoxidation and cyclizations to give rise to a variety of polycyclic triterpenoids, including cycloartenol. Cycloartenol, after a series of three demethylations (two at C-4 and one at C-14), opening of the 9,19-cyclopropane ring, and modifications to the side chain, gives rise to cholesterol, sitosterol, and campesterol. Stigmasterol is derived from sitosterol. Modified from Nomura *et al.* (1999).

4.2. Major Steps in Brassinosteroid biosynthesis

Campesterol (24 α -methylcholesterol) is the starting point for BL biosynthesis. The first important step, accomplished via two intermediates, is the reduction of the 5,6 double bond in campesterol to give rise to campestanol (Fig. 9-5). Subsequently, there are two parallel pathways from campestanol to CS, the immediate precursor of BL. In the early C-6 oxidation pathway, campestanol is oxidized to 6-oxo-campestanol, which then undergoes C-22 and C-23 hydroxylations in the side chain, followed by an epimerization of C-3-

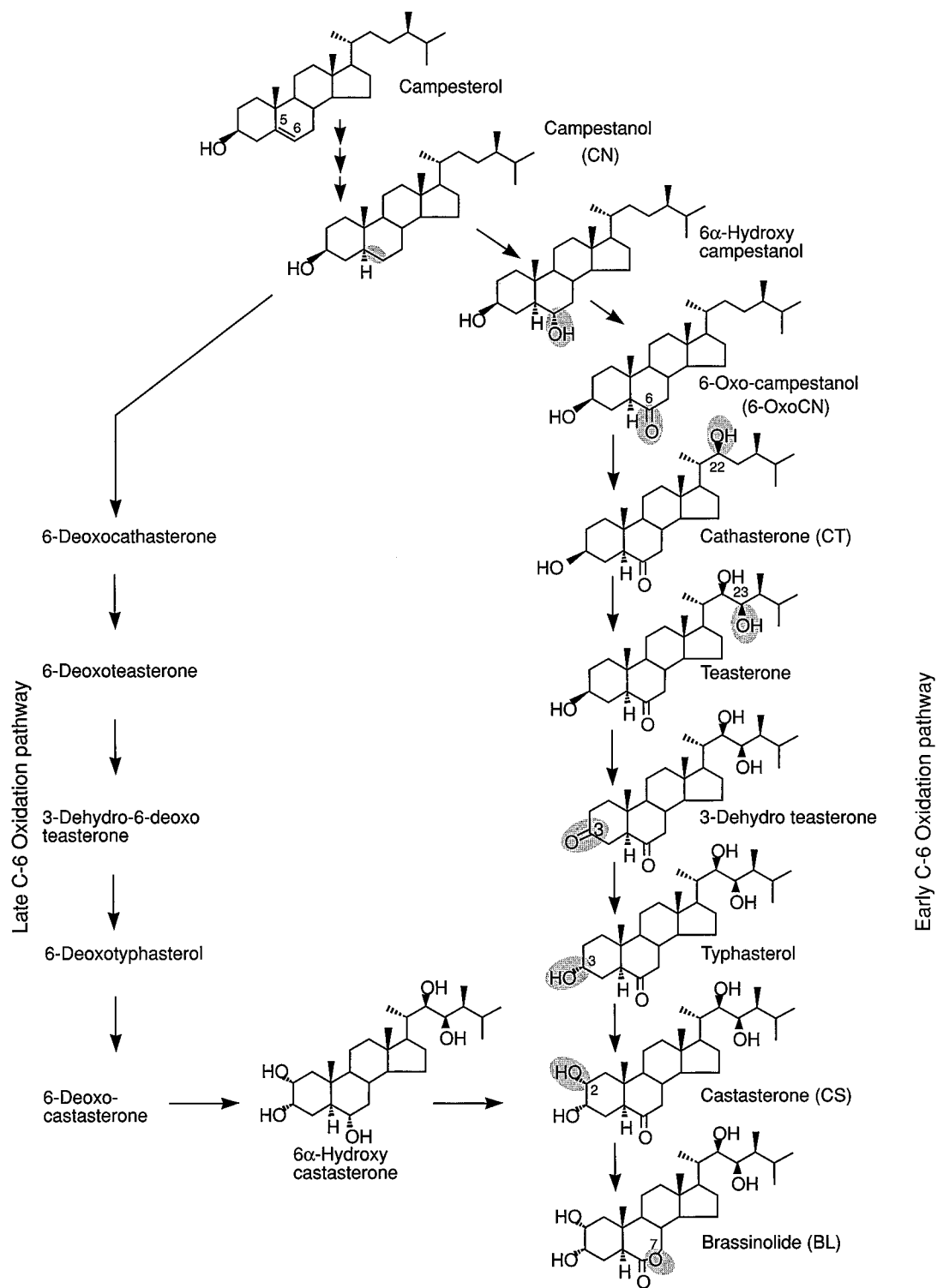


FIGURE 9-5 Biosynthetic pathways for brassinolide (BL). Campesterol undergoes reduction of the 5,6 double bond to form campestanol. Campestanol, by two alternate pathways, an early or a late C-6 oxidation, gives rise to castasterone (CS). Note that the C-3 epimerization between teasterone and typhasterol occurs *via* a 3-dehydro intermediate. CS is converted to BL via Baeyer-Villiger type oxidation. Modified from Choe *et al.* (1998) and Nomura *et al.* (1999).

hydroxyl and an addition of a C-2-hydroxyl in the A ring to give rise to CS. In the other pathway, the late C-6 oxidation pathway, campestanol, by similar hydroxylations and epimerization, gives rise to 6-deoxocasterone, which then gives rise to CS. In the final step, CS is converted to BL via Baeyer–Villiger-type oxidation.

Although the pathway just described was elucidated using *Catharanthus* culture cells, some steps have been confirmed in other species. Also, the widespread occurrence of the intermediates in other plants, such as tobacco, rice, *Arabidopsis*, and lily pollen, indicates that it is of general occurrence. The late C-6 pathway is more prevalent in a number of species including *Arabidopsis* and pea, while in tomato it appears to be the only route to BR production. It is possible that these pathways are regulated in a species or tissue-dependent manner; also, that they are environmentally regulated.

In some species, such as mung bean (*Vigna radiata*), tobacco (*Nicotiana tabacum*), and tomato (*Lycopersicon esculentum*), BL has not been found and CS seems to be the final product, whereas in others, such as *Catharanthus*, *Arabidopsis*, and possibly rice (*Oryza sativa*), BL is the final product. Thus, there seems to be some specific preference whether CS is the ultimate product or whether it is further oxidized to BL.

5. SYNTHESIS MUTANTS AND THEIR WILD-TYPE GENES

Enzymes catalyzing the various steps in BL biosynthesis have not been purified biochemically, but the genes encoding many of them have been isolated and cloned via analysis of BR synthesis mutants. Because one of the major biological activities of BRs is to promote stem and petiole elongation, mutants in BR biosynthesis can be screened by searching for the dwarf phenotype in mutagenized populations. It should be recalled that a dwarf phenotype is obtained in several GA-deficient mutants (see Chapter 7). It is also obtained in several mutants that show the morphology of light-grown seedlings when grown in dark (see Chapter 26). It is also seen in several mutants that are insensitive to auxin or ethylene in their response to these hormones (see Chapters 21 and 22). Thus, to unequivocally identify a dwarf mutant as one that is defective in the BL biosynthetic pathway requires many other tests, after the initial screening, including its sensitivity to other hormones and a determination of the endogenous content of CS, BL, and/or other intermediates. Other tests, such as complementation of the

loss-of-function mutants by transgenic expression of the wild-type gene, usually follow for confirmation.

Such rigorous screening and testing have led to the identification of several mutants that are blocked in the synthesis of campesterol or, in subsequent steps, in BR biosynthesis. These mutants are shown in Fig. 9-6.

The mutant *det2* was first identified as a mutant in the response pathway for light-mediated morphogenesis (see Chapter 26), but later was shown to be a BR synthesis mutant. The *lkb* mutant in pea was earlier thought to be a GA synthesis mutant. All mutants are dwarfs, which are restored to wild type when supplied with exogenous BRs, but not when supplied with exogenous

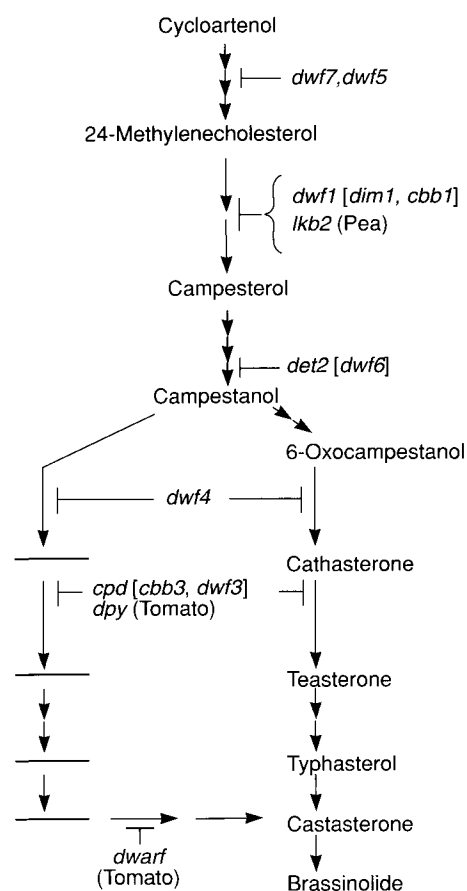


FIGURE 9-6 Sterol and brassinosteroid synthesis mutants. Note that the *dwf4* and *cpd* mutants affect both early and late 6-oxidation pathways. All are *Arabidopsis* mutants, except *lkb*, which is a pea mutant, and *dpy* (for droopy) and *dwarf*, which are tomato mutants. The *Arabidopsis* mutants were identified in several laboratories and, subsequently, some were shown to be allelic to others; the name that has precedence, according to the rules of nomenclature, is given first; others are indicated in square brackets. *dwf*, dwarf; *dim*, diminuto; *cbb*, cabbage; *det*, de-etiolated; *cpd*, constitutive photomorphogenic and dwarf. Adapted with permission from Clouse and Sasse (1998), Li and Chory (1999), and Bishop *et al.* (1999)

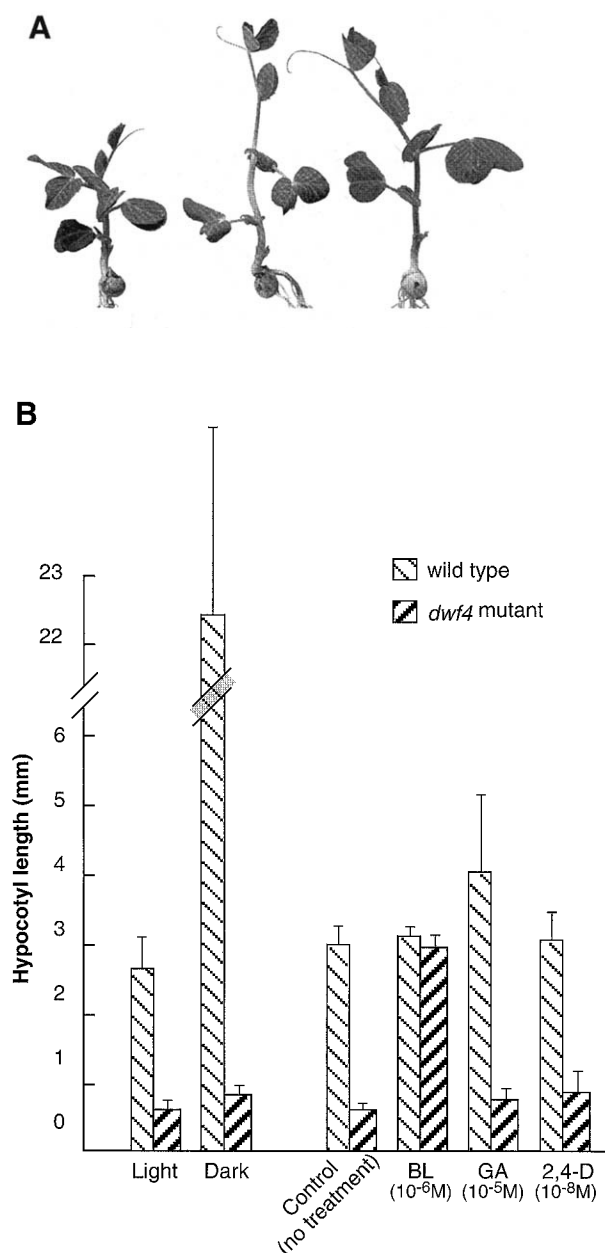


FIGURE 9-7 BR synthesis mutants. (A) The *lkb* mutant in pea (*Pisum sativum*). Left, *lkb* mutant; center, *lkb* mutant supplied brassinolide (BL, 100 ng); and right, wild type. Plants were grown for 3 days after the application of BL. Modified from Nomura *et al.* (1997). (B) The *dwf4* mutant of *Arabidopsis*. Data from two separate experiments are shown. The set on the left shows wild-type and mutant plants grown in light and dark (shaded). It is clear that the mutant is inhibited in growth both in light and in dark. The set on the right shows rescue of the mutant by BR, but not by GA or by the auxin, 2,4-D. Lines above the bars represent 1 SD. Modified from Azpiroz *et al.* (1998).

GA or auxin (Fig. 9-7). Conversely, GA synthesis mutants (e.g., *ga1* in *Arabidopsis*) are not rescued by an exogenous supply of BRs, which shows clearly that

while the two hormones control cell elongation, they do so via separate signaling pathways.

Wild-type genes of most of the BR biosynthesis mutants identified to date have been cloned and functionally characterized. *DWF1* and *DWF7* encode enzymes in the synthesis of campesterol from cycloartenol *via* 24-methylenecholesterol. *DWF1* is involved in both the isomerization and reduction of the $\Delta^{24(28)}$ bond, whereas *DWF7* is a Δ^7 sterol C-5 desaturase. *DET2* encodes a 5 α -reductase, which catalyzes the last step in the reduction of campesterol to campestanol, whereas *DWF4* and *CPD* encode cytochrome P450-type monooxygenases, which are involved in 22 α - and 23 α -hydroxylations, respectively. The *DWARF* gene in tomato also encodes a P450 monooxygenase, which catalyzes the conversion of 6-deoxoCS to CS.

6. INHIBITORS OF BRASSINOSTEROID BIOSYNTHESIS

As mentioned earlier in connection with GA biosynthesis (Chapter 7), the oxidation of *ent*-kaurene to *ent*-kaurenoic acid is mediated by cytochrome P450-type monooxygenases. These monooxygenases are diverse members of a large family encoded by multiple gene families. Many are inhibited in their action by triazole-type inhibitors, such as uniconazole and paclobutrazol (see Fig. 7-11, Chapter 7). The deduced amino acid sequences of *DWF4* and *CPD* proteins show similarity to that of D3, a P450-type monooxygenase, catalyzing a step in GA biosynthesis in maize (see Table 7-1). Uniconazole is reported to inhibit BL biosynthesis, although it is not known whether it specifically inhibits the 22 α - and/or 23 α -hydroxylations catalyzed by *DWF4* and *CPD*, respectively. Paclobutrazol has also been reported to inhibit the growth of barley seedlings. In this instance, inhibition is thought to be due to a lack of sterol synthesis because it is relieved by the application of stigmasterol.

7. BRASSINOSTEROID STRUCTURE AND BIOLOGICAL ACTIVITY

Several intermediates in the early C-6 oxidation pathway to BL show activity in bioassays, and the level of activity is generally related to the order in which they appear in the synthetic pathway. Thus, BL has the highest activity among naturally occurring BRs, followed by CS, which requires about five times

as much concentration as BL to give the same amount of activity; earlier intermediates show lesser activities than CS. The assumption, therefore, is that the specific features of the CS or BL molecules, namely 6-ketone or 7-oxalactone, and the two pairs of vicinal (neighboring) hydroxyls, i.e., the C-2 α - and C-3 α -OH in the A ring and the 22 α - and 23 α -OH in the side chain, are important for biological activity.

The 6-deoxoBRs generally have very low biological activity in most bioassays and often accumulate to large amounts relative to 6-oxoBRs. They have been considered to be a pool of inactive metabolites from which active forms could be derived. However, the situation is more complicated. In some bioassays, they are nearly as active as the 6-oxoBRs and, in some instances, show greater activity in light than in dark. It is possible that they are bioactive because they are converted to 6-oxo derivatives and that such conversion depends on the metabolic capabilities of species or tissue, which may be affected further by environmental factors, such as light.

The specific modifications in the side chain, i.e., a change from α to β configuration of -OHs and -CH₃ groups in the side chain, substitution of C₂H₅ for CH₃ at C-24, and so on, modify the biological activity.

8. REGULATION OF CASTASTERONE AND BRASSINOLIDE LEVELS

8.1. Regulation and Intracellular Site of Biosynthesis

Factors that regulate the biosynthesis of BRs in the plant are still mostly unknown. With the cloning of the genes encoding enzymes that catalyze steps in the synthesis of campesterol and intermediates in BL biosynthesis, the possibility is now open for studying the environmental and/or tissue-specific expression of these genes.

The regulatory steps are also unknown, although there are some leads. In *Catharanthus* cells, the endogenous levels of cathasterone (CT) are much lower than those of campestanol (CN), but the biological activity of CT is much higher than that of 6-oxoCN in the rice leaf lamina inclination assay. Accordingly, it has been proposed that the 22 α -hydroxylation of the side chain, catalyzed by the DWF4 monooxygenase, is a regulatory step (see Fig. 9-6). Another regulatory step is the conversion of 6-deoxocasterone (6-deoxoCS) to CS. The endogenous level of 6-deoxoCS is relatively high in several plants (e.g., *Catharanthus*, *Arabidopsis*), which suggests that it could be a "pool

BR," which is metabolized to CS and then BL, as needed. The cloning of the *DWARF* gene in tomato has confirmed this regulatory step.

8.2. Regulation by Deactivation

It is generally believed that BL or CS represents the final product in the synthetic pathway and is the biologically active compound. Some researchers still think, however, that there may be other subsequent products that have the same or even higher biological activity than BL. Assuming that BL or CS represents the final biologically active products, how are they deactivated?

Tritium-labeled BL or CS, or their C-24-*epi* forms, supplied to intact seedlings, excised stem segments, or cell cultures of monocot and dicot plants yields more polar as well as less polar products. Thus, BL and CS supplied to mung bean explants were converted to C-23 glucosylated products. 24-*epi*-BL fed to tomato cell cultures resulted in new hydroxylations at C-25 or C-26, followed subsequently by glucosylation of the newly formed hydroxyl groups (Fig. 9-8). A degradation of the side chain has also been reported.

It was mentioned earlier that sterol esters with fatty acids are common in membranes, especially plasma-lemma (see Section 4.1). Esters with fatty acids, such as lauric, myristic, or palmitic acids, have been reported for BRs in lily and in *Ornithopus* cell cultures. The 3 β -OH is used in this esterification; hence, it is preceded by epimerization of the C-3-OH (see Fig. 9-8).

Thus, exogenously supplied BRs undergo multiple reactions: hydroxylations and glucosylations in the side chain, reduction and side chain degradation, C-3 epimerization in the A ring, and conjugation with fatty acids. Many of the metabolites so formed are not found naturally *in planta*, and the role of these metabolic transformations in BR homeostasis is not clear. In lily, fatty acid conjugates were reported to release free BRs; thus, it is possible that these conjugates represent a way to store active BRs.

A gene encoding an enzyme that catalyzes the O-sulfonation at 22-OH of BRs has been isolated. Such sulfonation inactivates BRs, specifically 24-*epi*-cathasterone, in *Brassica napus*; and this type of inactivation is shared by estrogen activity in mammals. Interestingly, expression of the *Brassica* sulfotransferase gene was found to be induced by salicylic acid, a signal molecule in plant defense responses, which couples induction of the defense response with an inhibition of BR-mediated growth responses.

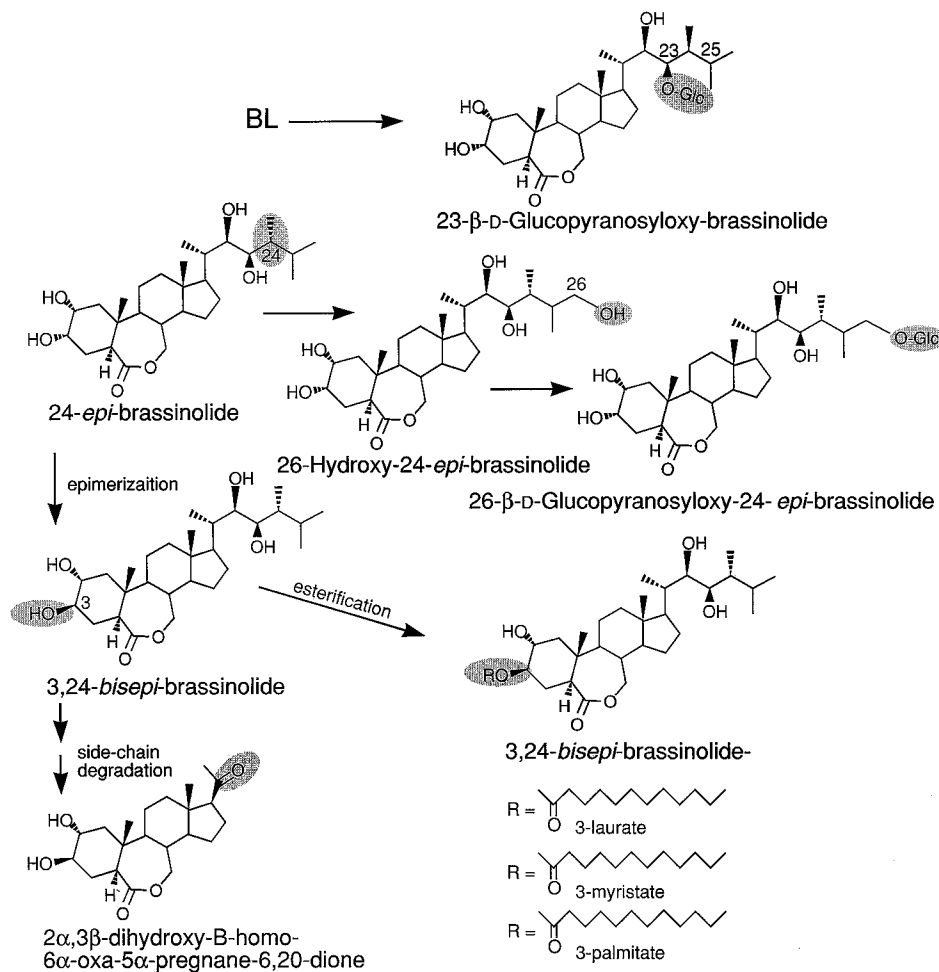


FIGURE 9-8 Some metabolites of BL and 24-*epi*-brassinolide. From Hai *et al.* (1995) and Kolbe *et al.* (1996).

9. CHAPTER SUMMARY

Brassinosteroids are a class of plant hormones with the A–D ring structure of steroids to which an alkyl side chain is attached. Many different BRs occur in plants, with the most potent ones being brassinolide and castasterone. BRs are widely distributed in the plant kingdom. In flowering plants they are present in nearly all parts and organs, with the highest concentrations in pollen grains and in immature seeds. Like other plant hormones, BRs serve multiple functions in growth and development, including elongation growth, promotion of male fertility, and promotion of senescence. They seem to have specific roles in elongation growth, both in light and in dark, and in counteracting the morphogenetic effects of light. The BR synthetic pathway has been elucidated using both labeled precursors and synthesis mutants. Starting from campestanol, which is derived from campesterol, two parallel pathways, one early 6-

oxoBR, the other 6-deoxoBR pathway, proceed to CS, which is converted to BL. Many genes, some encoding enzymes in campesterol biosynthesis and others encoding enzymes in the BR synthetic pathway, have been cloned. The possibility is now open for studying the environmental and/or tissue-specific regulation of synthesis of BRs, as well as the intracellular sites of their synthesis. Several metabolites of BRs have been isolated, including glucosylated derivatives, but their role in BR homeostasis is not well documented.

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Absciscic Acid

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1. DISCOVERY

In the 1950s and 1960s with the knowledge that auxins, gibberellins, and cytokinins were involved in cell division and/or cell growth, there was also the thought that there must be some natural hormones that act as inhibitors to growth. It was known that shoots stopped growing and became dormant in the fall/winter months in temperate climates, that mature

seeds became dormant, and that senescing leaves and mature fruits dropped off or abscised. Thus, extracts from several plant parts and tissues were tested for the inhibition of bud break, seed germination, IAA-induced growth of coleoptiles, and so on with positive results. Further fractionation and bioassays led to purification and crystallization in 1963 of “abscissin II” from fruits and leaves of cotton, which caused abscission of cotton fruits, and in 1965 of “dormin” from leaves of dormant sycamore (*Acer pseudoplatanus*) trees. The two substances proved to be chemically identical, and the substance was renamed abscisic acid (ABA) [for an interesting account of the discovery of ABA, see Addicott and Carns (1983)].

The name is a misnomer, however. Abscission of fruits and leaves is not one of the major functions of abscisic acid. Ethylene is the hormone that, in combination with auxin, regulates the abscission of leaves and fruits. ABA concentrations are high in cotton fruits, but abscission is related to its ability to stimulate ethylene production. ABA is involved, however, in many other important physiological processes (see Section 3).

2. STRUCTURE AND OCCURRENCE IN PLANTS AND FUNGI

ABA is a sesquiterpenoid (a C₁₅ terpenoid) with a ring and side chain (Fig. 10-1). There is an asymmetry at C-1' and thus there are (+) and (–) or (S) and (R)

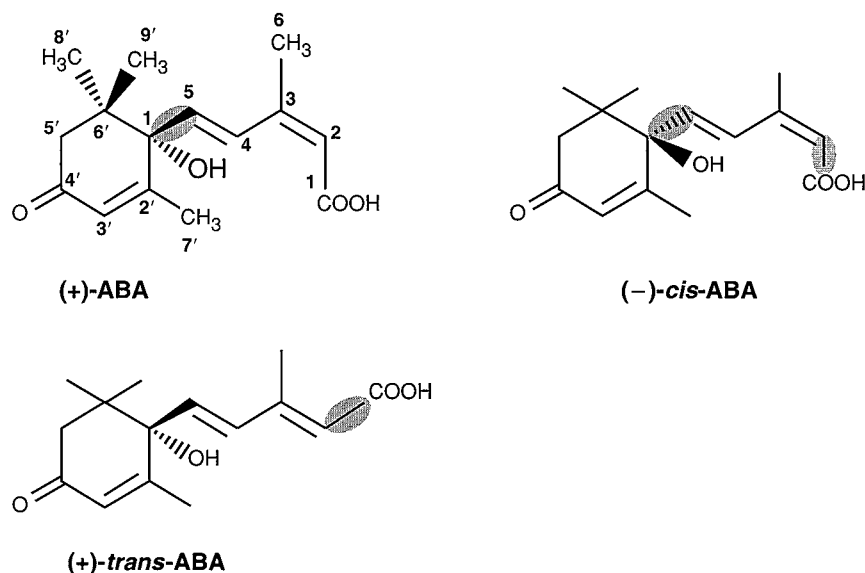


FIGURE 10-1 Structure of (+)-ABA [(+)-2-*cis*, 4-*trans*-abscisic acid] and enantiomers. The carbon numbering is shown.

forms. Only the S-(+) enantiomorph occurs naturally in plants. Commercial ABA is a racemic mixture of (+) and (-) forms and is written as (+/-)-ABA. Mirror-image pairs in the mixture form a more stable crystal structure with a higher melting point (190°C) than natural ABA (161°C).

In addition, there is an asymmetry at C-2 of the side chain. Thus, there is a *cis*, *trans*, and a *trans*, *trans* form. Most of the ABA present in plants is the 2-*cis*, 4-*trans* isomer. Only small amounts of the 2-*trans*, 4-*trans* isomer occur naturally. By convention, naturally occurring, (+)-2-*cis*, 4-*trans*-abscisic acid is called abscisic acid or simply ABA.

ABA is a weak acid and, at neutral pH in solution, occurs in a dissociated state; at acidic pH, it occurs in the protonated form. The protonated form diffuses freely across membranes, but the anionic form does not and requires active uptake *via* carriers (see Chapter 13). Depending on the pH in various cellular compartments, e.g., vacuole, cytoplasm, and chloroplasts, different amounts of ABA may be compartmentalized. Thus, ABA may be accumulated in chloroplasts, which have a higher pH than the cytoplasm.

ABA is found universally in plants—angiosperms, gymnosperms, ferns, horsetail (*Equisetum*), mosses, liverworts, and algae. Several species of phytopathogenic fungi produce ABA. Among them, *Cercospora rosicola*, which causes leaf spot disease in rose, and the related species, *C. cruenta*, have been used to study ABA biosynthesis, as they produce large amounts of ABA and release it in the culture medium.

3. PHYSIOLOGICAL ROLES OF ABSCISIC ACID (ABA)

Like other plant hormones, ABA has multiple functions. ABA seems to act as a general inhibitor of growth and metabolism, but these effects vary with tissue and developmental stage. ABA is present in many growing regions—young leaves may have more ABA than older, mature leaves, and its levels do not seem to be reduced in actively growing buds. Nonetheless, ABA applied to hypocotyls, epicotyls, leaves, and coleoptiles is generally inhibitory to growth. The effect of ABA on root growth is variable. In some cases it is inhibited, whereas in others it may be stimulated. Germination of most seeds and growth of excised embryos are inhibited by ABA. In nongrowing organs, application of ABA has been reported to inhibit protein synthesis and synthesis of nucleic acids, to affect membranes and levels of other plant hormones, and to induce abscission and senescence.

ABA increases the tolerance level of plants to various kinds of stresses caused by environmental or biotic factors. To give an example, plants growing in mesophytic climates are subject to sudden and unexpected periods of drought (water deficit or water stress) with an attendant danger of dehydration. Plants cope with this problem by increasing the levels of endogenous ABA quickly (Fig. 10-2), which, in turn, leads to closure of stomata to prevent water loss by transpiration and to synthesis of special proteins and compatible osmolytes (see Chapter 16) that protects cellular membranes and macromolecules from damaging

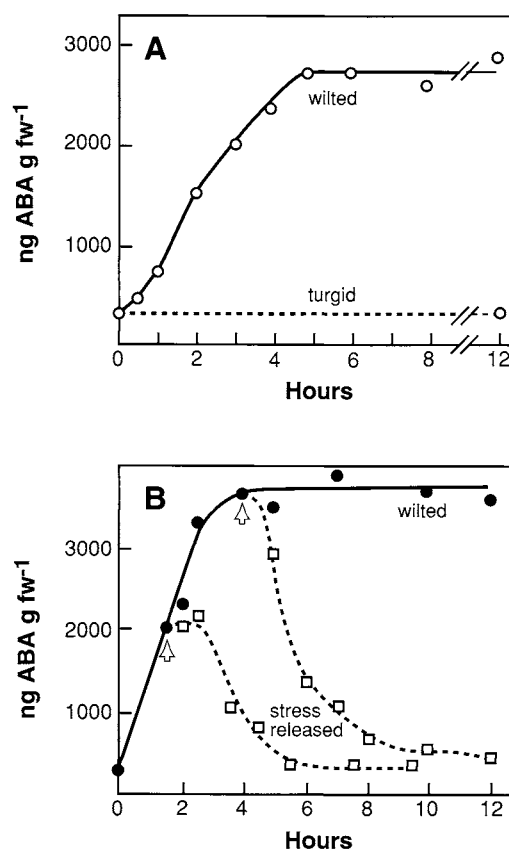


FIGURE 10-2 Accumulation of ABA in cocklebur (*Xanthium*) leaves by water stress and its loss following rehydration. (A) Detached leaves were allowed to wilt until fresh weight had been reduced by 10%. The ABA concentration increased steadily until plateauing after about 5 h. Comparative ABA concentrations in unstressed turgid leaves are shown. (B) In another experiment, leaves were allowed to wilt for 1.5 or 5 h before being rehydrated (open arrows) by submerging them into distilled water for 5 min. The ABA concentration declined almost immediately following rehydration. Modified from Zeevaert (1980).

effects of dehydration. On supply of water, the ABA concentration drops to normal.

Other stresses may be due to excessive salt content in the soil (salinity), heat, attack by pathogens and pests, and formation of reactive oxygen species when growing under low oxygen tension. Plants protect themselves against these stresses by several means, including synthesis of special proteins and osmolytes, and ABA is the chief hormone involved in such protection. For these reasons, it is often referred to as the "stress hormone."

ABA also plays a central role in seed development and maturation, enabling seeds to withstand desiccation and to become dormant. The role of ABA in the prevention of stress damage is covered in Chapter 16, and its role in seed development and maturation is covered in Chapter 18.

Some of the common bioassays for ABA are based on these responses. They include inhibition of seed germination, inhibition of α -amylase synthesis in aleurone tissue of cereal grains, stomatal closure in epidermal strips, and inhibition of elongation growth in stems and coleoptiles.

4. BIOSYNTHESIS OF ABA

The pathway for ABA biosynthesis was elucidated, as in the case of gibberellins, by a combination of biochemical and genetic techniques combined with physiological data.

As mentioned in Chapter 7, isopentenyl diphosphate (IPP) is the starting point for terpenoid biosynthesis. IPP isomerizes with dimethylallyl diphosphate (DMAPP), and further condensation of isoprene units with DMAPP gives rise first to geranyl diphosphate (GPP) and then farnesyl diphosphate (FPP) (see Fig. 7-5). Recall also that FPP, after another condensation with an isoprene unit, forms geranylgeranyl diphosphate (GGPP), which is the branch point for the formation of cyclic diterpenes (e.g., *ent*-kaurene and gibberellins), linear diterpenes (e.g., phytol), and, after dimerization, tetraterpenes (e.g., carotenoids). Carotenoids and their oxygenated derivatives, xanthophylls, provide an important backdrop for this chapter. Some aspects of their synthesis and role are highlighted in Box 10-1.

Farnesyl diphosphate (FPP) is the first sesquiterpenoid (C_{15}) compound and it was thought for some time that it could give rise directly to ABA after oxidation and cyclization. However, because the end groups of C_{40} carotenoids, such as violaxanthin, had a striking similarity to ABA, an alternate view was that ABA could arise indirectly by cleavage of a carotenoid precursor. These two possibilities are shown in Fig. 10-5.

Support for an FPP origin came from ABA biosynthesis in the fungus *Cercospora*, where ABA is formed from FPP *via* 1'-deoxy-ABA (Fig. 10-6). (Why fungi synthesize ABA is an unanswered question. It is speculated that an increased concentration of ABA may hasten the senescence of leaves and inhibition of protein synthesis, which in turn may favor susceptibility to the fungal pathogen.)

4.1. A Carotenoid Pathway for ABA Biosynthesis

In higher plants, however, the fungal pathway did not seem to prevail. Several mutants were known that were deficient in carotenoids and were also deficient

BOX 10-1 CAROTENOIDS AND THE XANTHOPHYLL CYCLE IN HIGHER PLANTS

CAROTENOIDS ARE AN ABUNDANT group of naturally occurring C_{40} pigments, which occur in thylakoid membranes in chloroplasts in association with chlorophyll-protein complexes; some also occur in the plastid envelope. Carotenoids also occur in chromoplasts and are responsible for the yellow to red colors of many flowers, fruits, and roots, such as carrot. (The other major class of pigments for red, yellow, and blue coloration of flowers and some other plant parts is anthocyanins, which occur in a soluble state in vacuoles). Carotenoids that have only carbon and hydrogen (hydrocarbons) are called carotenes, whereas derivatives containing oxygen functions are called xanthophylls. Carotenoids may be acyclic (e.g., lycopene) or contain five- or six-membered rings at one or both ends of the molecule (e.g., β -carotene, lutein) (Fig. 10-3). Because of the extensive double bond systems, a carotenoid molecule absorbs light and can act as a photoreceptor (see Chapter 27); it can also, in theory, exist in a large number of geometric isomers (*cis/trans* isomers). Most carotenoids are found in the all-*trans* form, whereas some exist as *cis* isomers. All-*trans* isomers are usually listed only by their names, whereas *cis* isomers are identified by the *cis* prefix.

Carotenoid synthesis proceeds by a two-step condensation of two molecules of GGPP to form pre-phytoene phosphate and then phytoene, the first C_{40} carotenoid (see Fig. 7-5). The enzyme phytoene synthase is located in the plastid stroma. Lycopene (responsible for tomato fruit coloration) is formed from phytoene via four stepwise dehydrogenations catalyzed by membrane-bound desaturase enzymes in plastids. Cyclization reactions lead to the formation of β -carotene (also α -, Ψ -, and δ -carotenes). Xanthophylls with oxygen substituents on the ring include lutein and zeaxanthin with hydroxy groups and violaxanthin and neoxanthin with epoxy groups (see Fig. 10-3).

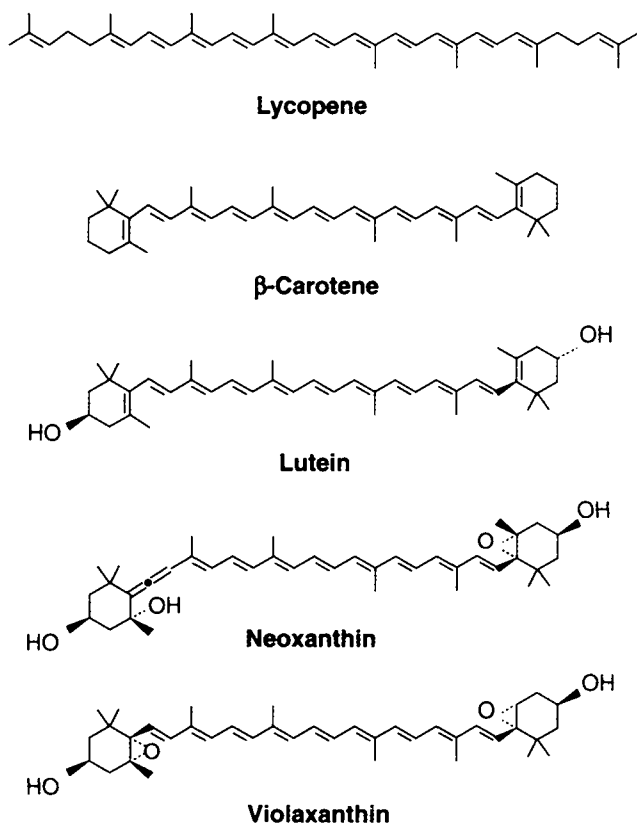


FIGURE 10-3 Typical carotenoids of higher plants. β -Carotene, lutein, violaxanthin, and neoxanthin are the most abundant carotenes/xanthophylls in photosynthetic tissues. Minor components include α -carotene, zeaxanthin, and antheraxanthin. Reproduced with permission from Bramley (1997).

Carotenoids serve two major functions in higher plants. As accessory pigments, they absorb light in the UV-A/blue regions of the spectrum and pass the light energy to chlorophyll. They also protect the chlorophylls from destruction under high light intensities by dissipating the excess energy (they quench the triplet state of chlorophyll [^3Chl] and scavenge for singlet oxygen [$^1\text{O}_2$], a reactive oxygen species that can cause peroxidation of membrane lipids). Three xanthophylls, zeaxanthin, antheraxanthin, and violaxanthin, are the principal xanthophylls involved in photoprotection. These three xanthophylls occur in thylakoid membranes and participate in a cyclic reaction known as the **xanthophyll cycle**. In this cycle, zeaxanthin by two successive epoxidation reactions is converted to violaxanthin, and the latter by two successive de-epoxidations is converted back to zeaxanthin. Anthraxanthin is the intermediate in each case (Fig. 10-4). Zeaxanthin epoxidase (ZE) and violaxanthin de-epoxidase (VDE) are the enzymes that catalyze these interconversions. The activities of these enzymes are regulated by pH and light conditions. ZE activity is favored under limiting light; hence, under darkness or low light conditions, the accumulation of violaxanthin is favored. Excessive light has the effect of raising the proton concentration in the thylakoid lumen, thus increasing the pH gradient (ΔpH) across the thylakoid membrane. Under these conditions, the activity of VDE is favored and zeaxanthin accumulates. Zeaxanthin is the xanthophyll involved in dissipation of excess light energy.

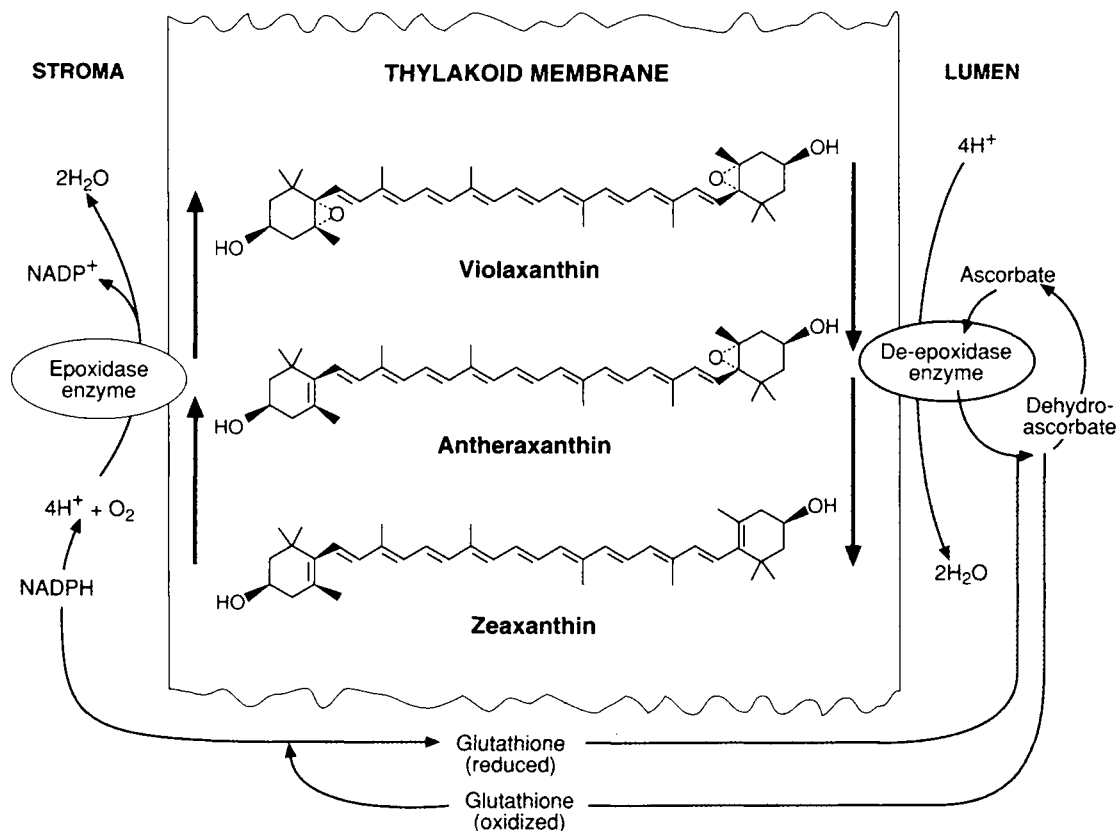


FIGURE 10-4 The xanthophyll cycle in higher plants. Zeaxanthin epoxidase has a pH optimum of 7.5, which prevails in the plastid stroma; hence the epoxidation reaction is believed to occur at the thylakoid membrane interface with the stroma. The violaxanthin de-epoxidase has a pH optimum of 5.1, which is also the pH of the thylakoid lumen; hence, the de-epoxidation reaction is believed to occur at the thylakoid membrane and thylakoid lumen interface. The epoxidation reaction requires NADPH and O_2 , whereas the de-epoxidation reaction requires ascorbate, which is converted to dehydroascorbate. In a complex series of oxidation-reduction reactions involving glutathione, NADPH and ascorbate are regenerated from NADP^+ and dehydroascorbate, respectively. From Boyer and Leegood (1997).

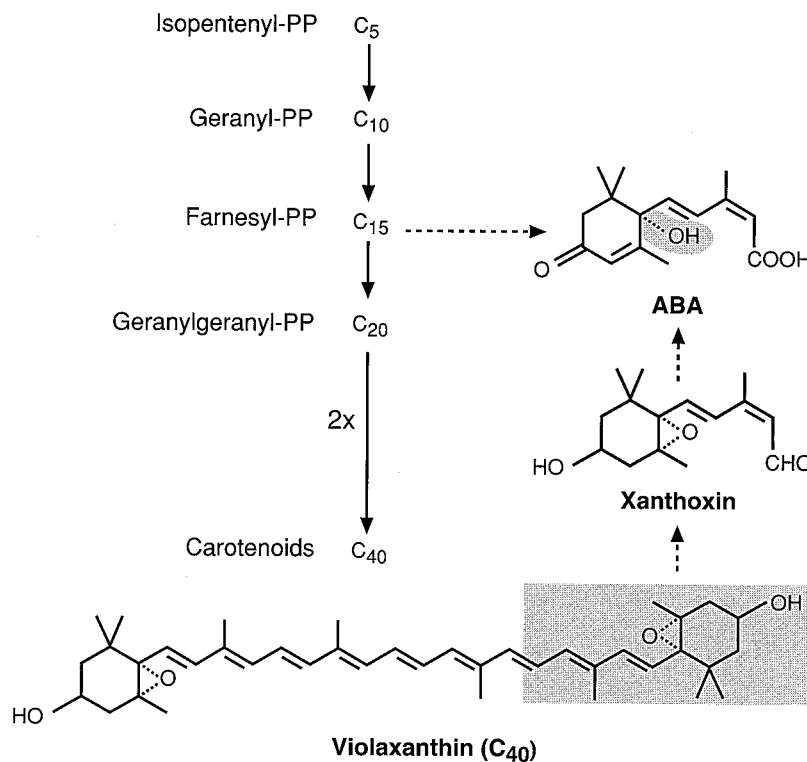


FIGURE 10-5 Two possible pathways for the synthesis of ABA in higher plants are shown by dashed lines, one directly from farnesyl diphosphate and the other an indirect pathway *via* carotenoids. Note the structural similarity between the end groups in violaxanthin (one group is shaded) and ABA. Modified from Walton and Li (1995) with kind permission from Kluwer.

in endogenous ABA (see Section 5). Also, the use of carotenoid synthesis inhibitors, such as fluridone or norflurazon (see Section 6), could, under certain circumstances, lead to a decline in endogenous ABA, whereas in the fungus, it did not inhibit ABA accumulation. Feeding experiments with radiolabeled precursors did not resolve the issue in higher plants as they did in fungi because the amount of radioactivity incorporated into ABA from labeled precursors, such as mevalonic acid (one of the two sources of isopentenyl diphosphate, see Chapter 7, Fig. 7-6), carotenoids, or CO₂, was poor. Another complicating factor was that the endogenous concentrations of ABA are small

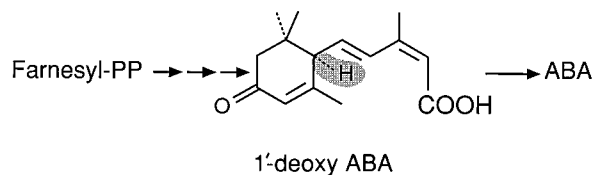


FIGURE 10-6 Synthesis of ABA in *Cercospora rosicola* from farnesyl diphosphate *via* 1'-deoxy ABA. Compare the shaded area in 1'-deoxy ABA with that in ABA in Fig. 10-5.

relative to the abundant pool sizes of the presumed precursors, carotenoids. Under these conditions, incorporation of the label in the product could not be adequately correlated with a decline in the label in the precursor.

In this dilemma, an experiment by Creelman and Zeevaart (1984), using the heavy isotope of oxygen, ¹⁸O₂, provided the needed breakthrough. It was known from physiological experiments that plants that are deprived of water and allowed to wilt accumulate large amounts of ABA (see Fig. 10-2). Accordingly, these authors allowed bean (*Phaseolus*) and cocklebur (*Xanthium*) plants to wilt while exposing the leaves to ¹⁸O₂. Because 1'-deoxy-ABA in *Cercospora* was the immediate precursor of ABA (see Fig. 10-6), it was expected that ¹⁸O would be incorporated in the hydroxyl group at the C-1' position in the ring. Surprisingly, however, ¹⁸O was not incorporated in the ring, but in the -COOH group in the side chain. This observation, while negating a derivation of ABA from 1'-deoxy-ABA, as in the fungus, suggested the possibility that a xanthophyll, such as violaxanthin, was cleaved oxidatively to form a product with an aldehyde group containing ¹⁸O and that the ring oxygens

were coming essentially unchanged from the precursor. The aldehyde, so formed, would be xanthoxin, and if xanthoxin were oxidized further, the result would be ABA with ^{18}O in the carboxyl group. The other oxygen atom in the COOH group would come from water (see Fig. 10-7). Subsequent ^{18}O labeling experiments with many other tissues, turgid or

stressed leaves, roots, ripening fruits, and etiolated leaves have confirmed this labeling pattern. The oxygen at C-1' also gets labeled with ^{18}O , but only in long-term labeling experiments and is due to turnover of the xanthophyll pool.

Further support for a carotenoid pathway for ABA synthesis in plants was provided by experiments in

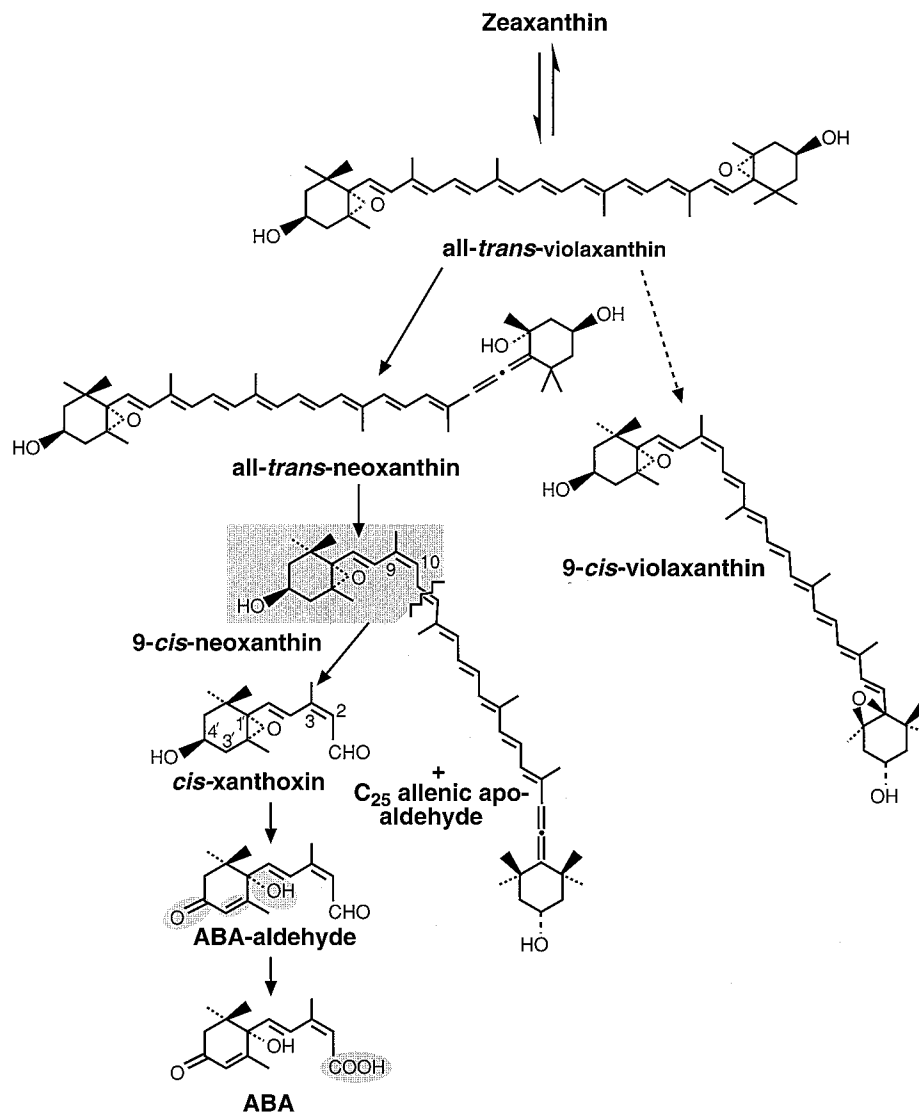


FIGURE 10-7 ABA biosynthetic pathway in higher plants. Zeaxanthin is epoxidated to yield violaxanthin. Violaxanthin gives rise to 9'-*cis*-neoxanthin, which is oxidatively cleaved to give rise to xanthoxin. A zigzag line indicates the site of cleavage. The latter compound gives rise to ABA-aldehyde, which in turn is oxidized to ABA (modifications during this derivation are highlighted). Note that for the natural isomer of ABA to be produced, xanthoxin has to be in the *cis* configuration; *trans*-xanthoxin has no biological activity, nor is it able to give rise to *cis*-xanthoxin or ABA. *cis*-Xanthoxin can only be obtained from a precursor that has a *cis* configuration corresponding to the 2,3 double bond of xanthoxin (i.e., the 9,10 double bond of the C_{40} carotenoid). Such a carotenoid could be 9'-*cis*-violaxanthin, but that compound occurs in small amounts and would require a very high turnover rate during the stress-induced synthesis of ABA. The other alternative, which was preferred, was that violaxanthin, which occurs in great abundance, was the precursor and gave rise to 9'-*cis*-neoxanthin *via* an intermediate, all-*trans*-neoxanthin. Modified from Walton and Li (1995) with kind permission from Kluwer.

which the carotenoid content was kept low by growing seedlings in darkness and by treatment with carotenoid biosynthesis inhibitors, fluridone or norflurazon. At the same time, the seedlings were water stressed to raise the endogenous content of ABA. The combined protocol, in effect, reduces the ratio of endogenous carotenoids to ABA and, thus, allows measurement of changes in their respective amounts. Using such seedlings, it could be shown that the increase in levels of ABA and its metabolites was accompanied by a 1:1 stoichiometric decrease in the total amount of xanthophylls, mainly violaxanthin and neoxanthin (Table 10-1).

Roots generally do not synthesize large amounts of carotenoids (only about 0.1 to 0.2% of those in light-grown leaves), and they can be put under water stress to enhance the synthesis of ABA. In these roots also, a loss of violaxanthin and neoxanthin with a corresponding increase in ABA content could be demonstrated.

Based on these data, the biosynthetic pathway for ABA in higher plants was proposed in 1990 independently by Daniel Walton, State University of New York, Syracuse, and Roger Horgan at University College of Wales, Aberystwyth, and their associates (Fig. 10-7). The scheme proposed the conversion of violaxanthin to 9'-*cis*-neoxanthin and the oxidative cleavage of 9'-*cis*-neoxanthin to form a C₁₅ aldehyde, *cis*-xanthoxin (called xanthoxin), and a C₂₅ apoaldehyde. Xanthoxin was the penultimate precursor and gave rise to ABA after ring modifications and further oxidation of the aldehyde group to the carboxyl group.

4.2. Epoxy-xanthophylls Are Required for the Synthesis of ABA

Two ABA-deficient mutants, *aba1* (for abscisic acid deficient) of *Arabidopsis* and *aba2* of tobacco (*Nicotiana glauca*), are defective in the epoxidation of zeaxanthin (see Fig. 10-8). In these mutants, zeaxanthin

accumulates, but the contents of epoxy-xanthophylls (violaxanthin and neoxanthin), as well as ABA, are low. The *ABA2* gene from tobacco has been cloned. It is homologous to its counterpart gene in *Arabidopsis* and, expressed as a transgene, is able to rescue both mutants. When expressed in *Escherichia coli*, the fusion protein shows zeaxanthin epoxidase activity *in vitro*. These data confirm that the epoxidation of zeaxanthin, or the presence of epoxy-xanthophylls, is a necessary condition for the synthesis of ABA. Whether the lesion in these mutants is between zeaxanthin and antheraxanthin or between antheraxanthin and violaxanthin is not known (see Fig. 10-4).

4.3. Cleavage of *cis*-Neoxanthin Is the Key Step in ABA Biosynthesis

The final piece in the carotenoid pathway for ABA synthesis fell in place with the cloning of the gene for the cleavage enzyme. A mutant in maize, *vp14*, shows a normal content of epoxy-carotenoids, but is still deficient in ABA. Because the mutant can convert xanthoxin to ABA aldehyde and to ABA (see Fig. 10-7), the likely site of the lesion would be the enzyme catalyzing the cleavage of the C₄₀ precursor of xanthoxin (C₁₅) and the C₂₅ apoaldehyde. The wild-type *VP14* gene was cloned by transposon tagging. The amino acid sequence of the encoded protein shows similarity to bacterial dioxygenases, and the *VP14* fusion protein expressed in *E. coli* catalyzes the cleavage of 9-*cis*-epoxy-carotenoids to form C₂₅ apoaldehydes and xanthoxin (C₁₅). The enzyme is specific to *cis* configuration; carotenoids in the all-*trans* configuration are not cleaved. This is as expected because the cleavage of 9-*cis*-epoxy-carotenoids produces *cis*-xanthoxin, which is in turn converted to the active isomer of ABA. Cloning of the *VP14* gene and functional characterization of the encoded protein as a nine-*cis*-epoxycarotenoid dioxygenase (NCED) was a crucial step in the carotenoid-derived biosynthesis of ABA.

TABLE 10-1 Effects of Water Stress on Decrease in Levels of Individual and Total Xanthophylls and Increase in Total ABA^a

Stress time (min)	9'- <i>cis</i> -Neoxanthin	Violaxanthin (Decrease nmol.g fw ⁻¹)	Total Xanthophylls	Total ABA (increase nmol.g fw ⁻¹)
40	-0.3	0.7	0.4	0.9
90	2.6	8.8	12.1	7.4
270	3.6	17.6	22.1	21.2

^aFor data in this table, bean (*Phaseolus vulgaris*) seedlings were grown for 8 days in the dark with fluridone treatment from days 4 to 8. Total xanthophylls include 9'-*cis*-neoxanthin, violaxanthin, 9'-*cis*-violaxanthin, and antheraxanthin. Total ABA includes ABA plus its two metabolites, phaseic acid and dihydrophaseic acid (for metabolites, see Section 7.3). From Walton and Li (1995).

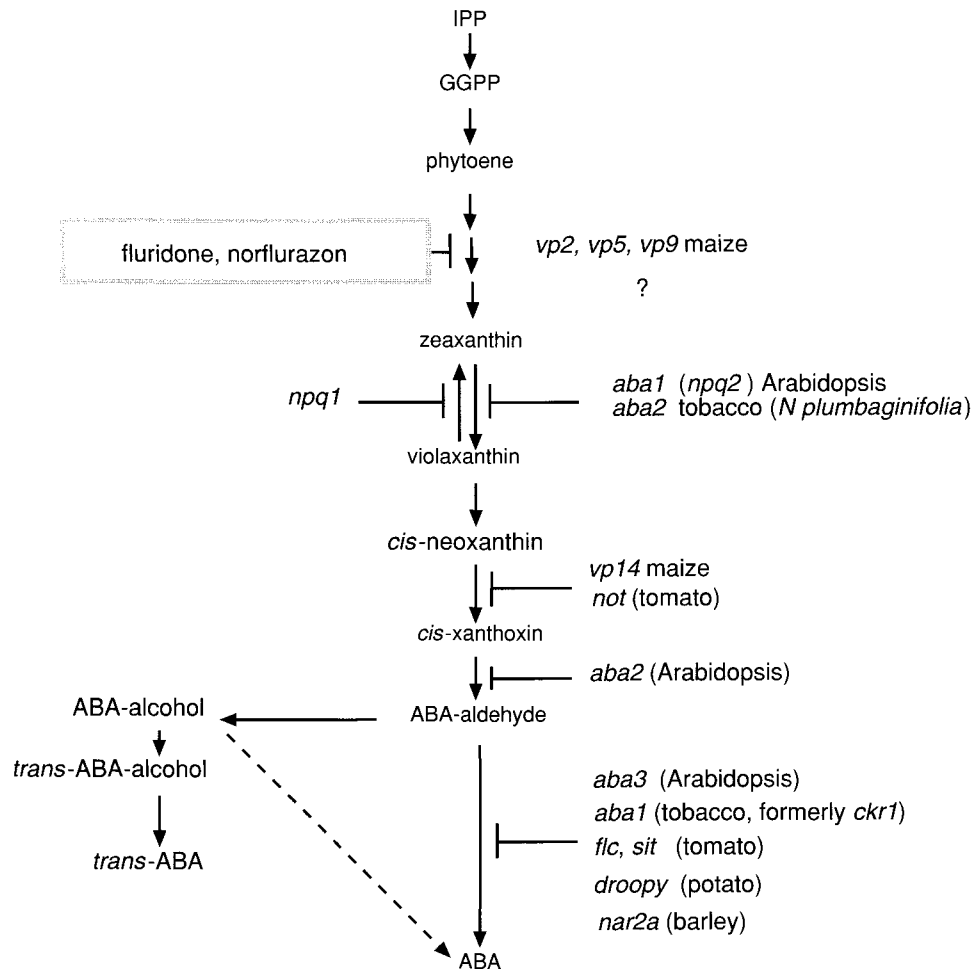


FIGURE 10-8 Carotenoids and/or ABA synthesis mutants and sites of their lesions. The *Arabidopsis* mutants, *npq1* and *npq2* (for nonphotosynthetic quenching), are defective in violaxanthin de-epoxidase and zeaxanthin epoxidase, respectively; *npq2* is allelic to *aba1*.

The *notabilis* (*not*) mutant in tomato (*Lycopersicon esculentum*) is an ABA-deficient mutant, which had remained uncharacterized until recently. Following cloning of the *VP14*, it was possible to isolate the *NOTABILIS* cDNA and show that it encodes a protein with sequence similarity to the *VP14* protein and likely a NCED (see Fig. 10-8). A cDNA encoding a similar protein has been cloned from bean (*Phaseolus vulgaris*) and others are likely to follow (see Section 7).

4.4. Conversion of Xanthoxin to ABA Is Catalyzed by Enzymes That Are Constitutively Expressed

If radiolabeled xanthoxin is supplied to plant tissues, it is converted to ABA, irrespective of whether the plant tissue is water stressed or not. Also, this

conversion is not subject to inhibition by inhibitors of transcription or translation. These data indicate that the last two steps in the synthesis pathway, the conversion of xanthoxin to ABA aldehyde and then to ABA, are catalyzed by enzymes that are constitutively expressed in plant tissues. As a result of such constitutive expression, the concentration of xanthoxin in plant tissue is kept low relative to that of ABA, and the supply of xanthoxin becomes the limiting factor in ABA biosynthesis. This limitation in the supply of xanthoxin has important implications in the regulation of ABA synthesis, as shown later (see Section 7.1).

4.5. Sites of Synthesis

ABA is synthesized in mature leaves, stems, developing seeds and fruits, roots, and indeed most

parts of the plant. Carotenoids in plants are restricted to chloroplasts and other types of plastids, such as etioplasts and chromoplasts. These are also the intracellular sites for the synthesis of isopentenyl diphosphate and carotenoids (see Fig. 7-6). Thus, the steps to 9'-*cis*-neoxanthin (see Fig. 10-7) occur in the plastids. The NCEDs in maize, tomato, and bean identified to date carry an N-terminal sequence that targets them to plastids; thus the cleavage enzyme is probably located in the plastids; but whether the cleavage reaction occurs in the plastids or the cytosolic side of the plastid envelope is unknown. Enzymes for the conversion of xanthoxin to ABA have not yet been purified or their genes cloned, but this conversion is believed to occur in the cytoplasm.

In summary, there is conclusive evidence that ABA is formed in higher plants from carotenoids. The evidence comes from labeling patterns of ^{18}O incorporation in ABA in plant tissues that are actively synthesizing ABA; from a demonstration of a 1:1 relationship between a decline in the content of xanthophylls and an increase in the content of ABA and its metabolites in plant tissues, which are low in carotenoid content; from a demonstration that in mutants deficient in epoxy-xanthophylls and in ABA, the levels of these compounds can be restored to wild-type levels by transgenic expression of zeaxanthin epoxidase; and finally by the cloning of the gene that encodes the cleavage enzyme and the demonstration that it is specific for 9-*cis*-epoxycarotenoids. However, the carotenoid pathway may not be the only and exclusive pathway for ABA synthesis in higher plants. The *aba2* mutant of tobacco produces 23–48% of the wild-type content of ABA, which is surprising because the gene is supposed to be a single copy. Therefore, it is possible that other less specific enzymes also carry out the epoxidation reaction or, alternatively, that ABA is produced by some other pathway as well.

5. CAROTENOID AND/OR ABA SYNTHESIS MUTANTS

ABA-deficient mutants have a tendency to wilt easily when they are under water stress because they are unable to close their stomata. Seeds of these mutants also lack dormancy and germinate readily when placed in water. Some show vivipary, i.e., seeds germinate while still on the mother plant (see Chapter 18).

In addition to the mutants referred to in Section 4 (*aba1* of *Arabidopsis*, *aba2* of tobacco, *vp14* of maize, and *not* of tomato), many other mutants in carotenoid and/

or ABA biosynthesis are known (see Fig. 10-8). Several *viviparous* (*vp*) mutants of maize (e.g., *vp2*, *vp5*, *vp7*, *vp9*) are deficient in xanthophylls and ABA. The exact sites of lesions are unknown, but are believed to be at some step earlier than zeaxanthin. One mutant, *vp1*, has normal xanthophyll and ABA content, but shows insensitivity to endogenous ABA, particularly in seed tissues. The *vp1* mutant is an ABA response mutant and is covered in Chapter 23.

The *aba2* mutant of *Arabidopsis* is blocked in the conversion of xanthoxin to ABA aldehyde; so far, it is the only known mutant for this step. As shown in Fig. 10-7, three modifications to the ring structure occur in this step: oxidation of the 4'-hydroxyl to a ketone, desaturation of a 2'-3' bond, and opening of the epoxide ring. They are all believed to be catalyzed by a single enzyme, xanthoxin oxidase, but the wild-type gene has not been cloned.

For the final step, oxidation of ABA aldehyde to ABA, many mutants are known [e.g., *flacca* (*flc*) and *sitiens* (*sit*) in tomato, *droopy* in potato (*Solanum phur-eja*), *aba3* in *Arabidopsis*, *aba1* (formerly *ckr1*) in tobacco, and *Az34* or *nar2a* in barley]. In this group of mutants, under water stress, ABA aldehyde (which would be expected to accumulate) is converted to *trans*-ABA aldehyde and then to *trans*-ABA alcohol, which accumulates. The low basal levels of ABA, which nonetheless are formed in these mutants, may arise from isomerization of *trans*-ABA alcohol to the *cis* form. Also, the *trans*-ABA alcohol may give rise to some *trans*-ABA. The lesion in some of these mutants (e.g., *nar2a* in barley, *flc* in tomato, *aba3* in *Arabidopsis*, and *aba1* in tobacco) is not in the aldehyde oxidase apoprotein, but in the molybdenum-binding cofactor that is required by the enzyme. The site of lesion in the *wilty* mutant in pea is unknown.

Mutants in the last two steps of ABA biosynthesis may be considered "true" ABA synthesis mutants because the carotenoid complement in these mutants is normal. These mutants are especially useful in deciphering the physiological roles of ABA in stress tolerance, stomatal closing, and seed maturation and dormancy.

6. ABA SYNTHESIS INHIBITORS

There are no known inhibitors for ABA biosynthesis *per se*. As mentioned earlier, fluridone and norflurazon inhibit carotenoid biosynthesis, probably the formation of C_{40} xanthophylls from phytoene by dehydrogenases (see Fig. 10-8), and, hence, indirectly can lower the ABA content. However, it should be empha-

sized that plants grown in light synthesize large amounts of carotenoids to act as accessory pigments and to protect the chlorophylls from photobleaching. Treatment of these plants with fluridone is generally not sufficient to lower the ABA content measurably because of the large pool of epoxy-carotenoids. Even for dark-grown plants, the continued use of fluridone for long periods may be necessary to lower the carotenoid pool size sufficiently to have an effect on ABA content. This problem is alleviated to some extent by using plant materials that are naturally deficient in carotenoids, such as roots or etiolated plants.

7. REGULATION OF ABA LEVELS

The endogenous levels of ABA are regulated both by synthesis and by conjugation or oxidation to inactive forms.

7.1. Regulation of Synthesis

As shown earlier, ABA content is increased rapidly when plants are subjected to an environmental stress, such as a water deficit (see Fig. 10-2). The question arises as to what step in ABA biosynthesis is upregulated by water stress? It appears that the cleavage step that leads to the formation of xanthoxin from 9'-*cis*-neoxanthin is the main regulatory step. This assumption is based on the reasoning that, in normal plants, the carotenoid precursors, violaxanthin and 9'-*cis*-neoxanthin, are always present in great abundance, whereas the levels of xanthoxin are kept low because the enzymes that catalyze its conversion to ABA are expressed constitutively.

With the cloning of the *VP14* gene from maize and homologous cDNAs from tomato (*NOTABILIS* or *LeNCED1*) and bean (*PvNCED1*) and characterization of their encoded proteins as NCEDs, it is possible to test the assumption just given. The mRNAs of all three clones are strongly induced under water stress. For bean, it has also been shown that the increase in mRNA and the NCED protein is correlated with an increase in the content of ABA (Fig. 10-9). Furthermore, it is known that inhibitors of transcription or translation (e.g., actinomycin D or cycloheximide) inhibit the stress-induced synthesis of ABA, but, as indicated earlier, they do not inhibit the conversion of xanthoxin to ABA. Thus, there is biochemical as well as physiological evidence for the regulation of ABA biosynthesis by induction of the cleavage enzyme.

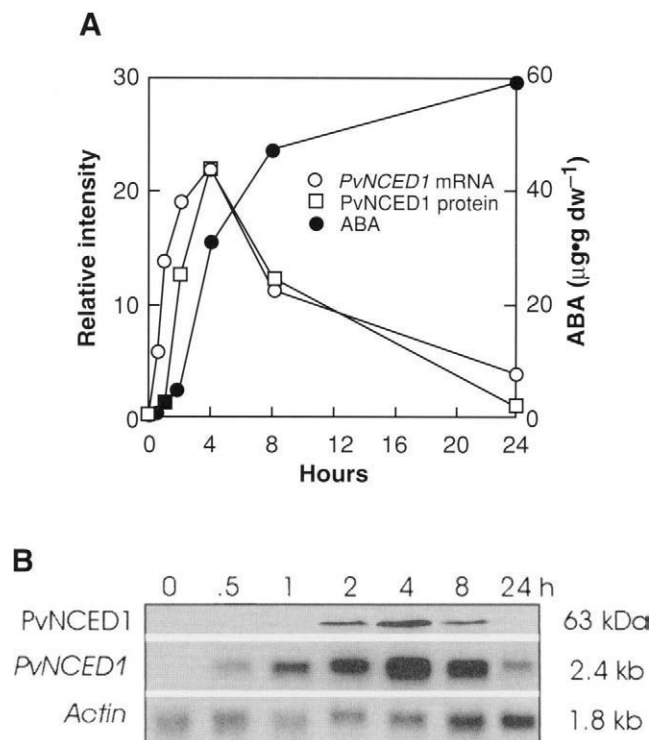


FIGURE 10-9 Effect of dehydration on transcript and protein levels of *PvNCED1* and ABA accumulation in detached leaves of bean (*Phaseolus vulgaris*) leaves. (A) Time course of changes. Note that the increase in *PvNCED1* mRNA and *PvNCED1* protein occurs rapidly and after reaching a peak their levels decline, while the ABA content continues to increase. (B) Protein gel blot cross-reacted with antiserum raised against recombinant *PvNCED1* protein (top) and RNA gel blot analysis of the expression of *PvNCED1* (middle) or *Actin* (bottom) as loading control. The ratio of *PvNCED1*/*Actin* is plotted in (A) as relative intensity. With permission from Qin and Zeevaart (1999), © National Academy of Sciences, USA.

How the cleavage enzyme is induced as a result of stress is unknown. In a critical study comparing three quantities—water potential, osmotic pressure (solute concentration) and turgor pressure—a loss of turgor pressure by cells was found to be most closely and directly related to ABA synthesis in drying leaf segments. It was also found that changes in leaf water potential played a role in the induction of ABA synthesis only if the turgor pressure had been reduced to less than 1 bar. Thus, loss of turgor and separation of plasma membrane from the cell wall may be the signal for perception of dehydration stress. We now know that the enzyme catalyzing the oxidative cleavage of 9'-*cis*-neoxanthin is the key reaction regulating ABA synthesis on drying, but the link between the separation of plasma membrane and the induction of the *VP14* gene in maize or its homologues in other plants is missing.

Several other DNA sequences related to *VP14*, *LeNCED1*, or *PvNCED1* have been identified in maize,

tomato, bean, and *Arabidopsis*. Not all of them encode the cleavage enzyme specific for ABA biosynthesis because they lack the targeting sequence for plastids or because their expression pattern has little to do with water stress. Nonetheless, it is possible that NCED enzymes in different plants are encoded by small multigene families, and individual members are expressed in response to developmental and/or environmental cues. This will undoubtedly be the subject for future investigation.

7.2. Inactivation of ABA

The biological activity of ABA has been related to its *cis* configuration, the -COOH group in the side chain, the tertiary hydroxyl, and unsaturation in the aromatic ring. Alterations in any of these functions render ABA inactive, although a few exceptions have been reported. As with other plant hormones, conjugation with glucose and oxidation to inactive products are used for regulation of the endogenous concentrations of ABA.

7.2.1. Major Metabolites of ABA

Conjugation to glucose occurs at C-1 in the side chain to form ABA-glucosyl ester (ABA-GE). A minor pathway results in conjugation at C-1' in the ring and gives rise to ABA-glucoside (ABA-GS) (Fig. 10-10).

ABA is also oxidized to phaseic acid (PA), which, in turn, undergoes reduction at the 4'-keto to give dihydrophaseic acid (DPA) or *epi*-DPA. The provision of an -OH group at C-4' allows for conjugation with glucose and the formation of DPA- and *epi*-DPA-glucosides (DPA-GS and *epi*-DPA-GS).

ABA-GE, PA, DPA, and DPA-GS are the major metabolites when ABA is supplied to leaves or other plant parts. They are also the major metabolites when ABA, which is rapidly synthesized during stress, is dissipated on the release of stress. As to which metabolites predominate varies with species and developmental state. Thus, in *Xanthium*, ABA-GE is a major metabolite, whereas in bean and pea seedlings or in *Vicia faba* leaves, DPA is the major metabolite. DPA-GS is a major metabolite in soybean seeds, tomato plants, and sunflower embryos. Maize cells in suspension culture convert ABA to PA, and ABA-GE is scarcely formed. In addition to the major metabolites of ABA, some minor metabolites are also formed. They include the glucosyl esters of PA, DPA, and *epi*-DPA. A 7'-OH-ABA is also reported.

ABA-GE is more polar than ABA and is generally considered to be the form in which ABA is accumulated in vacuoles. Stress-related responses in plants are triggered when the concentration of free ABA reaches a certain threshold, which varies with tissues and plants. It was thought that ABA-GE might be hydrolyzed to provide for an increase in endogenous

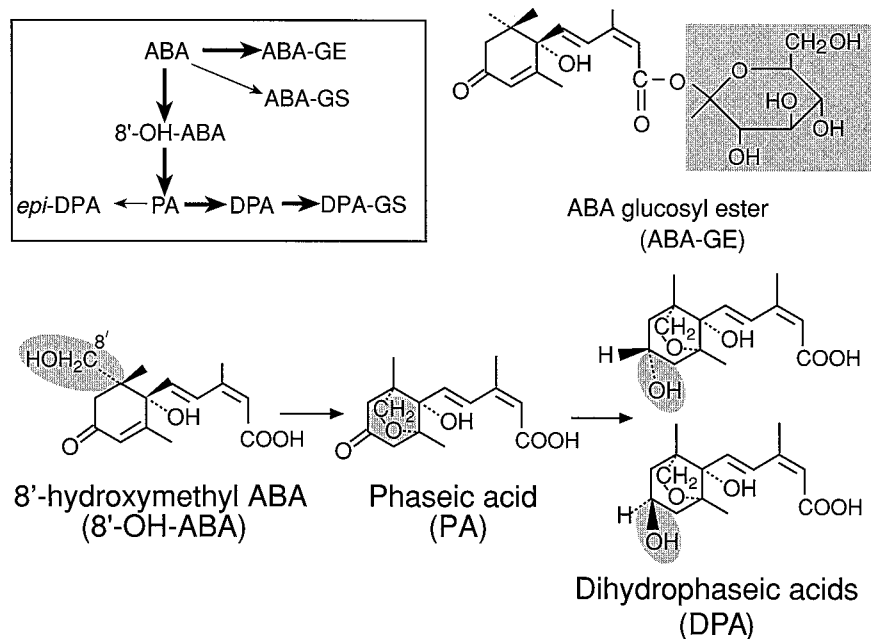


FIGURE 10-10 Some metabolites of ABA. Major pathways are shown by bold arrows. Adapted from Zeevaert (1999).

ABA when vegetative tissues are subjected to water stress. However, that is not the case. In a study where tomato plants were placed under water stress and induced to wilt, the levels of ABA rose sharply in leaves and then, on rehydration, declined sharply, but the amount of ABA-GE in the vacuoles remained constant or increased. Labeling experiments with ^{18}O indicate that ABA-GE is in fact synthesized at a slow rate, not hydrolyzed, when *Xanthium* leaves are subjected to water stress.

Although endogenous ABA-GE is not hydrolyzed to ABA in vegetative tissues, ABA-GE supplied to plant tissues is easily hydrolyzed to yield free ABA. Such hydrolysis probably occurs at the cell surface and may involve glucosidases rather than esterases. ABA then enters the cells and, subsequently, may be translocated in vascular tissues or conjugated and stored in vacuoles.

The situation in seed tissues may be different. ABA-GE accumulates in large amounts in maturing seeds and seems to be hydrolyzed when seeds imbibe water. Free ABA released from these esters is reported to inhibit seed germination (see Chapter 19).

7.2.2. Kinetics of Accumulation of ABA Metabolites during Stress

During dehydration of plant tissues, ABA synthesis is accompanied by an accumulation of PA (and DPA). Free ABA levels are regulated by the rate of ABA synthesis and by the rate of its degradation to PA. In early stages of stress, the rate of ABA synthesis is much higher than the rate of its conversion to PA, thus ABA accumulates. The rate of ABA conversion to PA, slow at first, gradually increases until it equals the rate of ABA synthesis ($\sim 5\text{--}6\text{ h}$ after the beginning of dehydration stress). The ABA concentration then plateaus at a high level, sufficient to trigger stress-related responses, and then declines. If the tissue is rehydrated, the rate of ABA decline is accelerated because synthesis rapidly drops to zero (within $\sim 1\text{ h}$), while the conversion of ABA to PA continues at a high rate until the concentration of ABA returns to normal low levels (Fig. 10-11). Subsequently, PA is also removed by conversion to DPA or *epi*-DPA, which may also be glucosylated.

As mentioned earlier, ABA-GE is also accumulated, but the rate of PA accumulation under stress is much higher than that of ABA-GE. Following rehydration, while the ABA and PA concentrations drop to normal levels, the drop in ABA-GE, in contrast, is insignificant, if it occurs at all (see Fig. 10-11). These data from kinetics of PA and ABA-GE accumulation during

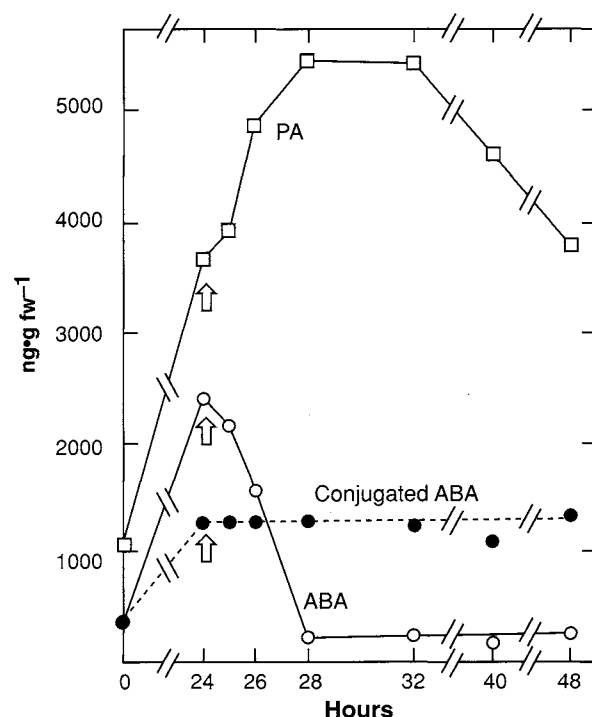


FIGURE 10-11 Kinetics of changes in ABA, PA, and ABA-GE in detached *Xanthium* leaves during stress and following rehydration. Leaves were rehydrated after 24 h of wilting. Note that the drop in ABA content follows almost immediately after rehydration, but the concentration of PA continues to increase for much longer until ABA has been reduced to normal levels. Conjugated ABA is ABA-GE. From Zeevaart (1980).

dehydration and loss after rehydration indicate that the oxidative pathway is the major pathway used for regulating free ABA content in abiotic stress.

7.2.3. The Oxidative Pathway for ABA Catabolism

The conversion of ABA to PA occurs *via* an intermediate, 8'-OH-ABA, which is unstable and rearranges to PA. The reaction catalyzed by ABA-8'-hydroxylase, which is expressed at high rates in various tissues recovering from water stress, is a major regulatory step in ABA homeostasis. Maize cells in suspension culture are a good system to study ABA metabolism. These cells do not have endogenous PA, but, as mentioned earlier, they synthesize PA on the addition of ABA and do not accumulate ABA-GE; moreover, PA can be quantitated readily because it diffuses out in the culture medium and samples can be collected for analysis. Using such a system, it has been shown that PA is synthesized rapidly on the addition of ABA and that such synthesis is blocked if cells are pretreated with transcription or protein synthesis inhibitors, cordycepin or cycloheximide, respectively. These data suggest that the gene encoding ABA-8'-hydroxylase is induced

de novo and the inducer is ABA. These observations explain the accumulation of PA in water-stressed leaves referred to in the previous subsection. They also provide a parallel situation to that for cytokinin oxidase, the principal enzyme responsible for the catabolic breakdown of endogenous zeatin and isopentenyl adenine, which is also induced by its substrate (see Chapter 8).

ABA-8'-hydroxylase is a cytochrome P450 monooxygenase. It should be recalled that enzymes in stage 2 of GA biosynthesis are also cytochrome 450 monooxygenases. Cytochrome P450 monooxygenases are a large superfamily of enzymes, which are involved in many metabolic pathways in plants. They also play a significant role in the detoxification of allelopathic substances and herbicides (see Chapter 6). They are integral membrane proteins of the endoplasmic reticulum that use molecular oxygen, require NADPH and NADPH-dependent cytochrome 450 reductase for activity, and are inhibited by CO. This inhibition is reversible by blue light. They are also inhibited, nonspecifically, by tetracyclis and triazole-type growth retardants. In the case of ABA-8'-hydroxylase, the use of tetracyclis results in inhibition of PA synthesis while ABA and ABA-GE accumulate. Attempts to purify and clone the gene for ABA-8'-hydroxylase are in progress.

Synthetic ABA analogs, which are altered in the 8'-OH and thus are immune to attack by ABA-8'-hydroxylase, are much more stable in plant tissues under stress conditions. They may also show a higher level of biological activity than ABA in bioassays.

The fates of compounds such as ABA-GE, DPA, and DPA-GS, which seem to be the end products of ABA metabolism, as also that of C₂₅ apoaldehydes released from oxidative cleavage of C₄₀ epoxy-xanthophylls, are not known. Most likely, they are broken down by various lipoxygenases and peroxidases in the cell.

7.3. Metabolism of (–)-ABA

Chiral HPLC columns can separate the two enantiomers of ABA (or PA). Thus, it is possible to obtain sufficient quantities of the two enantiomers and study their metabolism. Such studies are relatively few, but they indicate that (–)-ABA is metabolized at a much slower rate than natural (+)-ABA and it does not induce the activity of ABA-8'-hydroxylase in maize suspension cell cultures. The two forms of ABA also give rise to different metabolites. For instance, the (–)-form gives rise predominantly to ABA-GE, whereas the (+)-form gives rise to PA.

8. CHAPTER SUMMARY

Abscicic acid is a sesquiterpene, which has important roles in seed development and maturation, in the synthesis of proteins and compatible osmolytes, which enable plants to tolerate stresses due to environmental or biotic factors, and as a general inhibitor of growth and metabolic activities. ABA is derived in higher plants from a C₄₀ carotenoid, 9'-*cis*-neoxanthin, which is oxidatively cleaved to give xanthoxin (C₁₅) and an apoaldehyde (C₂₅).

Xanthoxin in a two-step process undergoes ring modification and oxidation of the side chain to give ABA. The last two steps in conversion of xanthoxin to ABA are mediated by enzymes that are constitutively expressed, whereas the cleavage reaction, catalyzed by a 9'-*cis*-epoxycarotenoid dioxygenase (NCED), is the main regulatory step in the synthesis of ABA. The carotenoid synthesis pathway starts from isopentenyl diphosphate, the precursor for all terpenoid syntheses, and occurs in plastids. The enzyme NCED is targeted to the plastids and the cleavage reaction occurs in the plastids or at the plastid–cytosol boundary. The last two steps in ABA synthesis probably occur in the cytoplasm.

This pathway, first worked out biochemically, is supported by the discovery of mutants that are impaired in the epoxidation of zeaxanthin or in the oxidative cleavage of 9'-*cis*-neoxanthin, and the cloning of the wild-type genes encoding the enzymes (ABA2 in tobacco and VP14 in maize, respectively). Other *viviparous* mutants in maize are impaired in carotenoid synthesis and, hence, in ABA content. The sites of their lesions are not precisely known. Many ABA synthesis mutants are defective in the last step, catalyzing the oxidation of ABA aldehyde to ABA; and one is defective in the previous step, catalyzing the conversion of xanthoxin to ABA aldehyde. These mutants in the last two steps could be considered as ABA synthesis mutants, as opposed to carotenoid synthesis mutants. They are proving useful in defining the physiological roles of ABA in plants.

The ABA content in plant tissues is regulated at the synthesis level by upregulation of the gene encoding the cleavage enzyme, NCED, and at the catabolic level by oxidation of ABA to PA mediated by the enzyme ABA-8'-hydroxylase. During stress and after relief of stress, ABA levels are precisely regulated by the differential between the rate of ABA synthesis and its conversion to PA. The differential is high in early hours after beginning of stress, then narrows, and finally turns negative. Thus ABA concentration in stressed tissue shows a steep rise, a plateauing, and a decline.

PA is further metabolized to dihydrophaseic acid (DPA), *epi*-DPA, and their glucosides. ABA is also inactivated by conjugation with glucose to form ABA-glucosyl ester (ABA-GE). ABA-GE is hydrolyzed *in vitro* to yield free ABA, but it is not the source of free ABA when vegetative tissues are subjected to water stress; rather ABA is synthesized *de novo* from carotenoid precursors. ABA-GE may be hydrolyzed in seed tissues, however, to yield free ABA.

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Ethylene

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1. DISCOVERY AS A HORMONE

That ethylene had morphogenetic effects on plants was discovered by accident in the closing years of the 19th century in a murky, gas-lit laboratory in the Botanical Institute at Petrograd, Russia. Pea seedlings were being grown in the dark, and it was noted by

Dimitry Neljubov that instead of being erect, long, and slender with a modest apical hook, these seedlings grew horizontally, were short and swollen, and had a much tighter apical hook. In a series of experiments in which he analyzed the constituents of the illuminating gas, Neljubov (1901) identified the culprit as the contaminant ethylene (C_2H_4). This effect of ethylene on dark-grown seedlings became known as the “triple response” (Fig. 11-1).

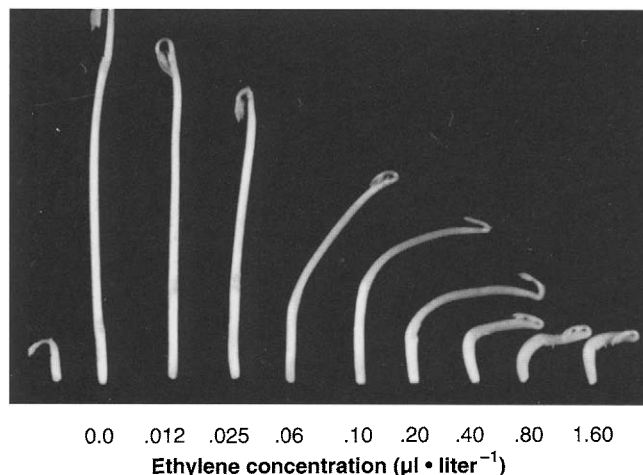


FIGURE 11-1 The effect of different concentrations of ethylene on the morphology of dark-grown pea epicotyls. Photograph taken after 48 h exposure to ethylene. The initial size of the seedling is shown on the left. Note that ethylene causes an inhibition of elongation growth, swelling of the stem (epicotyl), and tightening of the apical hook. In pea, and a few other plants, it also causes horizontal bending or “diageotropic” growth. Courtesy of S. F. Yang, J. D. Goeschl, and H. K. Pratt.

In the 1930s, it was established that ethylene was naturally produced by plant materials, especially ripening fruits, but whether ethylene caused ripening or whether it was a by-product was difficult to decide because methods to accurately measure ethylene, especially inside plant tissues, say a fruit, were not at hand. In the 1950s, use of syringes to pump out ethylene from plant materials and the advent of gas chromatography (GC) for identification and quantitation made it possible to measure small amounts of ethylene and led to its recognition as a plant hormone with a major role in fruit ripening.

2. STRUCTURE, DISTRIBUTION, AND INTERNAL CONCENTRATIONS

Ethylene, with a MW of 28, is one of the simplest organic molecules with biological activity. It is universally produced by all plants investigated; angiosperms, gymnosperms, ferns, mosses, and liverworts. It is also produced by a wide variety of fungi, yeast, and bacteria, including *Penicillium digitatum* (a citrus mold), *Cryptococcus albidus* (a yeast), and *Pseudomonas syringae* pv. Phaseolicol (a bacterium infecting *Pueraria labata*), which produce large amounts of ethylene in culture. Soil microorganisms account for much of the ethylene produced in the soil. In angiosperms, it is produced by essentially all parts of the plant: roots, stems, leaves, flowers, and fruits.

Ethylene concentrations are measured using GC equipped with a flame ionization detector. However, translation of these values to endogenous concentrations in a fruit or tissue usually involves a correction factor because ethylene, being a gas, diffuses out of plant tissues easily. The correction factor, known as **conversion constant**, is based on internal concentrations divided by production rates (see Table 11-1) and is determined by prior experimentation using the plant tissue and/or species of interest. Some of the highest production rates are recorded in the senescing flowers of the orchid (*Vanda*) and carnation (*Dianthus*), for some fruits during ripening, and for some fungi.

3. PHYSIOLOGICAL ROLES AND BIOASSAYS

Like other plant hormones, ethylene also has multiple functions. One of the best studied ethylene

TABLE 11-1 Ethylene Production Rates in Some Higher Plant Tissues and Fungi in Pure Culture

Tissue	Production rate (nl · g ⁻¹ · h ⁻¹)	Conversion constant (μl · liter ⁻¹ / nl · g ⁻¹ · h ⁻¹)
Climacteric fruits		
Avocado	0.02–130	0.7
Banana	0.02–20	1.9
Cherimoya	0.10–320	1.3–0.4
Cantaloupe	0.02–80	2.5
Tomato	0.02–20	2.0
Nonclimacteric fruits		
Lemon	0.02–0.1	1.8
Orange	0.02–0.1	4.0
Leaf		
Tobacco	0.50–2	0.1
Stem		
Bean	2	0.4
Flower		
Orchid (<i>Vanda</i>)	3400	
Carnation	0.05–30	
Fungi		
<i>Penicillium digitatum</i>	6000	
<i>Fusarium oxysporum</i>	3800	
f. sp. tulipae		

^aThe conversion constant is the ratio of internal ethylene concentration (μl · liter⁻¹) to the ethylene production rate (nl · g⁻¹ · h⁻¹). It is remarkably uniform within one plant tissue or organ, but varies between tissues/organs and with their physiological and developmental states. For climacteric and nonclimacteric fruits, see Section 3. Adapted from Beyer *et al.* (1984).

responses is in fruit ripening. Ripening of fruits involves many closely related changes: color change, softening of walls, and conversion of starch to sugar. Many fruits, such as banana, apple, avocado, and tomato, show what is known as a “climacteric,” a sudden and dramatic rise in respiration rate, which is accompanied or even preceded by C₂H₄ production, followed by fruit ripening (see Fig. 17-10). In these fruits, known as climacteric fruits, the massive and continued production of ethylene is the engine that drives the fruit-ripening process. Other fruits, known as nonclimacteric fruits (e.g., lemon, orange, and strawberry), show no climacteric and no significant production of ethylene, even though they show ripening-related changes. The role of ethylene in the ripening of climacteric fruits is covered in Chapter 17.

Ethylene promotes senescence and abscission of plant organs (e.g., leaves, flowers) and induces the synthesis of enzymes and defense- or pathogenesis-related (PR) proteins associated with these processes.

Senescence and abscission are covered in Chapter 20. Several stress situations, e.g., exposure of plants to heat or cold, wounding by mechanical means or *via* insect bites, attack by fungi and bacteria, and even gentle stroking of plant tissues, elicit ethylene production. In many of these situations, as in senescence and abscission, ethylene causes the production of PR proteins. Some of these responses are shared by ABA and jasmonates, which like ethylene are regarded as "stress" hormones, and it is not always clear which response is mediated by which hormone.

Flooding or submergence under water is a stressful situation because it results in the depletion of soil oxygen. Some plants (e.g., maize) respond to flooding by the enhanced production of lateral or adventitious roots; these roots develop large air spaces in the cortex (aerenchyma) for enhanced aeration. Such aerenchyma development can be induced by the exposure of plants to ethylene. In some plants that are native to habitats that are subject to periodical flooding (e.g., deepwater rice), a special mechanism is used to keep the aerial parts above water. The youngest stem internode has the capacity to elongate rapidly on flooding. In deepwater rice, this response is initiated by ethylene, but is mediated via ABA and gibberellin (see Chapter 15).

Exposure of plants to ethylene causes drooping of leaves and flowers, a phenomenon known as epinasty. Epinastic curvature results from unequal (or asymmetric) growth on the upper and lower sides of petioles or peduncles. The upper (adaxial) side grows at a faster rate than the lower (abaxial) side, resulting in a downward curvature of the organ (Fig. 11-2).

Asymmetric growth is also seen in the apical hook in hypocotyls or epicotyls of dicot seedlings grown in darkness and supplied ethylene — the adaxial side

grows faster than the abaxial side of the hook (see Fig. 11-1). In the field, in seedlings growing under soil, ethylene produced by the seedling causes the hook to be formed and maintained until the seedling pushes through the soil. The triple response, seen under laboratory conditions, is an enhanced stress response of seedlings germinating under soil that is tightly packed. On emergence from soil, the hook straightens because the abaxial side grows faster than the adaxial side. The asymmetric growth of axial organs is covered in Chapter 15.

Ethylene is implicated in many other physiological responses. Seedlings exposed to ethylene display an extraordinary production of root hairs, sometimes in root epidermal cells that normally do not produce them. Ethylene is reported to promote seed germination, cause sprouting of lateral buds in potato tubers, break dormancy, and cause accelerated growth and flowering in horticultural varieties of iris, narcissus, gladiolus, freesia, and other bulb crops. Ethylene is also reported to induce flowering in plants such as pineapple and mango.

The "triple response" of dark-grown seedlings is a common bioassay for ethylene. Reduced elongation and swelling by ethylene can also be induced in stems and petioles that are made to grow rapidly, either by growing them in the dark or treating them with hormones, such as GA. Other bioassays include ripening of climacteric fruits and measurement of epinastic curvatures. Ethylene is effective at low concentrations; many responses begin at 0.01 ppm and reach saturation at 1.0 ppm (1 ppm = $1 \mu\text{l.liter}^{-1}$ or 4.4 nM in H_2O).

4. BIOSYNTHESIS IN HIGHER PLANTS

The biosynthesis of ethylene has been studied mostly in ripening fruits of tomato, avocado, and melon and in senescing petals of the carnation, organs that produce abundant ethylene. Wounding of tissues and treatment with lithium chloride (LiCl) enhance ethylene production and sometimes are used in combination.

4.1. Major Steps in the Pathway

The ethylene biosynthetic pathway was essentially worked out in the late 1970s, chiefly by the efforts of Shang fa Yang and colleagues at the University of California, Davis, but the final steps in the synthesis were not clarified until recently.



FIGURE 11-2 The epinastic response to ethylene of tomato plants. From Lanahan *et al.* (1994).

The major steps are as follows:

- i. Methionine, a sulfur-containing amino acid, is converted to *S*-adenosyl-L-methionine (AdoMet or SAM) using ATP. The adenosine moiety of ATP is added to methionine with the release of PP_i and inorganic phosphate in a reaction catalyzed by Ado-met synthase (SAM synthase) (Fig. 11-3).
- ii. AdoMet is cleaved to give rise to 1-aminocyclopropane-1-carboxylic acid (ACC, a nonprotein amino acid) and methylthioadenosine (MTA). This reaction is catalyzed by a key enzyme, ACC synthase.
- iii. ACC is oxidatively decarboxylated by another key enzyme, ACC oxidase, to give rise to ethylene.
- iv. Methylthioadenosine (MTA) is cleaved by a nucleosidase to give rise to adenine and methylthioribose (MTR).

- v. MTR, by a complicated series of steps, regenerates methionine.

Methionine is in short supply in plant materials and, in certain situations, such as ripening fruits that are synthesizing ethylene rapidly, its supply can become limiting. This is prevented by conservation and recycling of the CH₃-S group of methionine. Use of ³⁵S- or ³H-labeled methionine has confirmed that MTA and MTR carry the CH₃-S group. Given a constant pool of the CH₃-S group and available ATP, a high rate of ethylene production can be sustained for a long time.

The breakdown of ACC to C₂H₄, mediated by ACC oxidase, requires molecular oxygen. HCN is a by-product; it is detoxified by conversion to β-cyanoalanine

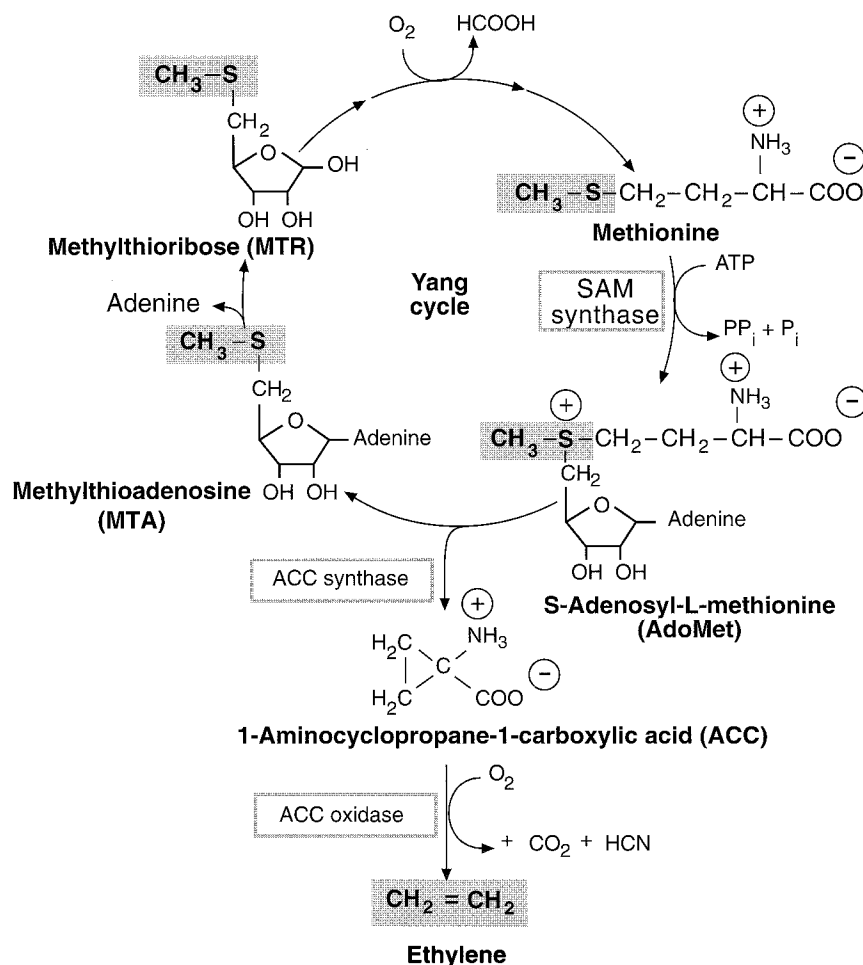


FIGURE 11-3 Biosynthetic pathway of ethylene in higher plants. Note that the CH₃-S group (bold face and shaded) of methionine is recycled back. The ACC oxidation to ethylene requires molecular oxygen, and the by-product, HCN, is detoxified to harmless products. The three enzymes in ethylene biosynthesis, SAM synthase, ACC synthase, and ACC oxidase, are indicated. Modified from McKeon *et al.* (1995).

and then to asparagine. This detoxification allows for continued synthesis of ethylene.

4.2. Exceptions to the Pathway

The pathway just described for ethylene biosynthesis is well established in angiosperms that have been investigated, but there may still be other pathways. For instance, water ferns (*Marsilea quadrifolia* and *Regnellidium diphyllum*) and liverwort (*Riella heliophylla*) produce ethylene from some precursors other than ACC. ACC supplied to fern leaflets is not converted to ethylene, nor do the inhibitors of ethylene biosynthesis, e.g., aminoethoxyvinylglycine or α -aminoisobutyric acid (see Table 11-2) inhibit ethylene production. Microbial organisms also produce ethylene by pathways different from the one in higher plants.

It should also be noted that ethylene in higher plants can be produced by the oxidation of many types of organic molecules, especially unprotected long chain fatty acids. Several enzymes, lipoxygenases, and peroxidases, can nonspecifically catalyze the oxidation and generate substantial quantities of ethylene. However, in all cases of stress-induced ethylene production in higher plants that have been studied, the pathway *via* SAM is utilized.

4.3. Ethylene Synthesis Is Induced under a Variety of Different Situations

Ethylene is produced at low basal rates throughout plant development, but its production is increased

sharply at certain stages, such as seed germination, ripening of climacteric fruits, and senescence of certain types of flowers, e.g., carnation and morning glory. Stresses of various kinds, chilling, heat, drought, flooding, radiation, wounding, treatment with metals such as copper and lithium, and attack by pathogens, all cause increased ethylene production, leading to the sobriquet, "stress hormone," or "stress ethylene." Treatment with hormones, such as auxin or cytokinin, also causes enhanced ethylene production. Most remarkably, ethylene not only induces its own synthesis, a phenomenon known as "autoinduction," but also inhibits its synthesis (autoinhibition) (see Section 6.2).

The induction of ethylene by many different types of inducers was a mystery that could not be explained until the cloning of the genes of the two key enzymes in ethylene biosynthesis: ACC synthase and ACC oxidase. For cloning a gene, usually the protein is purified, sequenced in whole or in part, degenerate oligonucleotide probes are prepared and used to screen a cDNA library. The isolated cDNA clone can then be used to isolate the gene from the genomic library. Thus, we progress from protein to gene. Alternatively, if synthesis mutants are known, it may be possible to clone the wild-type gene (see Appendix 1). For ethylene, however, synthesis mutants are not known (see Section 5); and purification of these enzymes, especially ACC oxidase, could not be accomplished by traditional biochemical methods. Alternate molecular techniques were needed. They are explained in Box 11-1.

TABLE 11-2 Characteristics of ACC Synthase and ACC Oxidase

Enzyme	~ M_r	Cofactors (cosubstrate)	Inhibitors
ACC synthase	50–60 kDa	Pyridoxal-phosphate	Aminoethoxyvinylglycine (AVG) Aminooxyacetic acid (AOA)
ACC oxidase (dioxxygenase)	35–36 kDa	Fe^{2+} (ascorbate)	Co^{2+} ; α -aminoisobutyric acid (AIB); Fe^{2+} chelators, e.g., 1,2-dihydroxynaphthalene, 1,10-phenanthroline (PA); EDTA

BOX 11-1 PURIFICATION OF ACC SYNTHASE AND ACC OXIDASE AND CLONING OF THEIR GENES

ACC SYNTHASE WAS DIFFICULT to purify because it occurs in very low amounts and is highly unstable. For its purification, therefore, it was essential not only to boost its relative amounts in a tissue, but also to develop a fast method for its purification. It was known that its synthesis could be increased by treatment with auxin or stress conditions, such as wounding, or treatment with LiCl (how LiCl promotes ethylene synthesis is not precisely known), but a fast purification protocol was still needed. Hans Kende's group at the Plant Research Laboratory, Michigan State University, East Lansing, MI, used pericarp tissue from ripening tomato fruits. These tissues produce large amounts of ethylene, but the synthesis was further enhanced by cutting the fruits in half, removing the pulp, and filling the emptied shells with LiCl for overnight treatment before extraction. A combination of HPLC and several other chromatographic procedures gave a sufficiently purified protein preparation, which could be used to prepare monoclonal antibodies (MAbs). These MAbs, in turn, were used to obtain a highly enriched enzyme fraction in one single step by mixing the crude enzyme preparation with MAbs bound to Sepharose beads and, subsequently, eluting the bound enzyme with a suitable buffer (see Chapter 5).

Cloning of a cDNA encoding ACC synthase also utilized the production of antibodies. Sato and Theologis (1989) used a mixture containing IAA and LiCl to induce ethylene synthesis in zucchini (*Cucurbita pepo*) fruits. They obtained a partially purified ACC synthase preparation and used it to generate polyclonal antibodies, which were further purified to give ACC synthase-specific Abs. A cDNA library derived from mRNA extracted from zucchini fruits was expressed in a bacterial vector, and the purified Abs were used to screen the expressed proteins. As a result, a cDNA clone could be selected, which, when expressed in yeast, provided functional confirmation that it was ACC synthase.

Although it was known that ethylene was formed by the oxidation of ACC and required molecular oxygen, the identity of ACC oxidase (ACO, formerly referred to simply as "ethylene-forming enzyme") remained a mystery for several years. Its discovery was a serendipitous outcome of research in fruit ripening. Donald Grierson and colleagues at the University of Nottingham, UK, were isolating cDNA clones that were specifically expressed during the ripening of tomato fruit. They identified several clones; some encoded known proteins (e.g., polygalacturonase), but the identity of others was unknown. One clone, called pTOM13, which was highly expressed, encoded a 35-kDa protein that was known from earlier experiments to be upregulated during ripening. To determine its function, these authors expressed the cDNA of pTOM13 in an antisense construct under the control of a constitutive promoter (CaMV S35) in transgenic tomato. The transformed plants showed much less expression of pTOM13 mRNA when green fruit or leaves were wounded, or during ripening of fruit than the non-transformed plants; they also showed much less production of ethylene (Fig. 11-4). The amino acid sequence predicted for pTOM13 showed a 58% similarity to the amino acid sequence of an enzyme, flavanone-3-hydroxylase. Based on these data, Grierson and colleagues suggested that pTOM13 could be coding for ACO.

Confirmation was provided by functional expression of the cDNA clone in two separate and independent transformations, one of yeast cells and the other of *Xenopus* oocytes. In each case, a known cosubstrate of ACO, ascorbate, enhanced the production of ethylene, and the known inhibitors of ACO, cobalt ion (Co^{2+}) and 1,10-phenanthroline (PA), inhibited its production. The gene was isolated by screening the genomic library with the cDNA probe. Because the predicted amino acid sequence for pTOM13 bore similarity to flavanone 3-hydroxylase, conclusive evidence was provided by extraction of a cytosolic enzyme from melon fruit, which could be assayed for ACO *in vitro* under conditions known to be optimal for flavanone-3-hydroxylase activity.

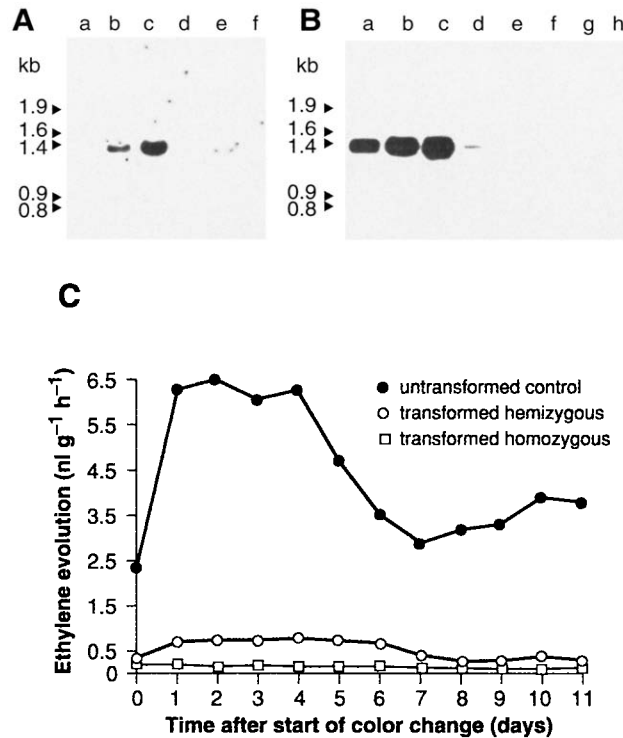


FIGURE 11-4 Expression of pTOM13 RNA in wounded leaves (A) and ripening fruits (B) of tomato plants transformed with an antisense construct of pTOM13 cDNA. Lanes on the left represent untransformed controls (in A, lanes a–c; in B, lanes a–d), whereas lanes on the right represent transformed plants. Samples of RNA, extracted from wounded leaves or fruits, were loaded (10 μg /lane) and hybridized to a sense-specific pTOM13 RNA probe. (C) Evolution of ethylene by detached fruits from untransformed and transformed plants. Ethylene production was measured by gas chromatography, and samples were taken for measurement daily for 11 days after the onset of color change. Plants grown from seeds derived from a selfed transformant yield progeny, which segregates for the inserted pTOM13 antisense gene. Segregates show 0, 1, or 2 copies of the inserted DNA. Plants with one or two copies of the inserted gene are referred to as hemizygous or homozygous, respectively. Reprinted with permission from Hamilton *et al.* (1990).

Cloning of ACC synthase and ACC oxidase genes clarified the nature of the two enzymes and their cofactor/cosubstrate requirements with much greater accuracy than was possible before. These details are summarized in Table 11-2. It also allowed a determination of the expression patterns of the two enzymes in different tissues and at different developmental stages, and the factors that may regulate them.

ACC synthase (ACS) is a soluble enzyme located in the cytoplasm and has a pI of 5.8 to 6.0. It is a labile enzyme with a half-life of ~ 58 min. The enzyme seems to occur as a dimer, but is catalytically active both as a dimer and as a monomer. The intracellular location of ACC oxidase (ACO) is not clear. The purified enzyme has no signal sequence or transit peptide and appears to be a soluble enzyme. However, for *in vitro* activity, an association with membrane fractions, possibly from tonoplast or plasmalemma, is required. Like ACC synthase, it is an unstable enzyme with a $t_{1/2}$ of 2 h

plus. In addition to Fe^{2+} and ascorbate, CO_2 is an essential activator of ACC oxidase.

4.4. ACC Synthase and ACC Oxidase Are Encoded by Multigene Families

ACC synthase genes (ACS) have been cloned from a number of plants (e.g., tomato, *Arabidopsis*, zucchini, winter squash, apple, potato, lupin). Several ACS genes occur in a species, each encoding an isoenzyme, thus there is a family of genes encoding ACC synthase in any one species. In tomato, nine ACS genes are known; in *Arabidopsis*, five of which three encode functional enzymes. Sequence analysis shows that these genes have several regions that are highly conserved, including the one that contains the catalytic site (Fig. 11-5), but they differ in the 5' promoter regions and in the 3' regions corresponding to the carboxy termini.

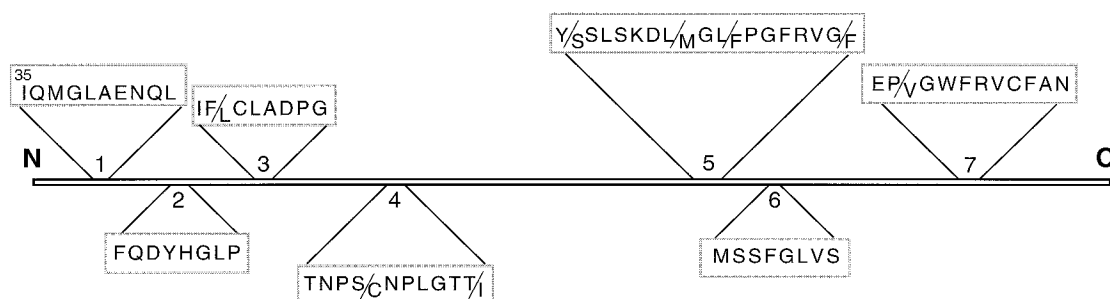


FIGURE 11-5 A structural map of ACC synthase based on a deduced amino acid sequence from a cDNA clone from apple. The seven highly conserved regions are boxed in, and consensus amino acids are shown by single letter designations; the active site resides in region 5. Adapted from McKeown *et al.* (1995).

Use of gene-specific probes shows that members of a family are expressed differentially in different tissues/organs and under different inducing conditions. Thus, in *Arabidopsis*, four ACS genes are expressed differentially (Fig. 11-6). ACS1/3 is expressed only under treatment with cycloheximide. ACS2 is expressed constitutively in all tissues, although its expression in stem tissues is less than in roots, leaves, flowers, or siliques (fruits). ACS2 is also maximally induced after wounding, under anaerobiosis, or after treatment of seedlings with LiCl, IAA, or cycloheximide (a potent protein synthesis inhibitor). ACS4 is expressed specifically by auxin. It is also expressed by cycloheximide, but much less by wounding. ACS5 is expressed similarly to ACS4 in all treatments, except for auxin or cycloheximide treatments. ACS5 has since been shown to be responsive to low levels of cytokinins in the seedling stage, not adult plants, and to be regulated mostly at the posttranscriptional level. A similar cytokinin modulation of auxin-induced ACS4 is also reported. Similarly, in tomato, different members of the ACS gene family are differentially regulated during fruit ripening, after auxin treatment, and after mechanical stress or wounding.

Nucleotide sequence analysis of these genes reveals another striking fact: ACS genes expressed in response to one stimulus, say auxin treatment, are more similar in amino acid sequence to each other among different species than they are to other ACS genes in the same species.

ACS genes provide an elegant example in plants of a common evolutionary theme, that of using nearly the same coding sequence with variations in the promoter region. This is an economical way to produce different isoforms of the same protein (in this case, an enzyme), but whose synthesis can be triggered by different developmental, environmental, or hormonal cues. While this adds a measure of complexity to understanding the signal transduction pathway, it

has immense survival benefit for the plant because it provides access to the same molecule *via* different routes. In addition, small differences in the coding sequence entail subtle functional differences in the proteins. For instance, different isoenzymes may differ in their K_m volume for substrate(s) or in pH or temperature optima to provide further, more specific, regulation.

ACS genes show another important feature of gene induction. Most members of the ACS gene family in a plant are also induced by cycloheximide, a feature typical of primary response genes (see Appendix 1).

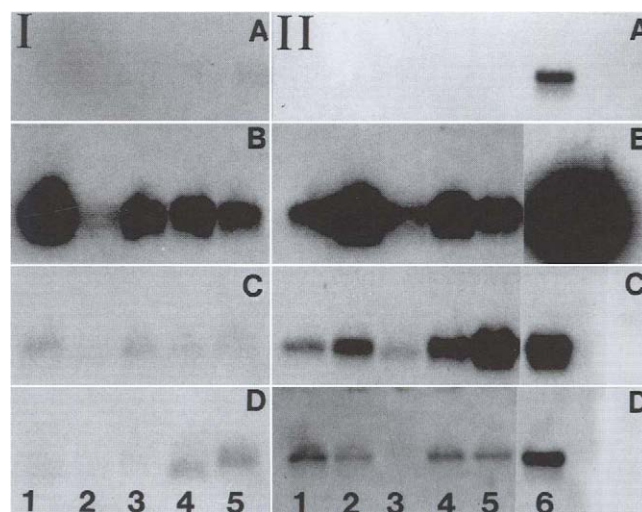


FIGURE 11-6 Differential expression of ACC synthase genes in *Arabidopsis*. (I) Expression of four ACC synthase genes—ACS1/3 (A), ACS2 (B), ACS4 (C), and ACS5 (D)—in different tissues. Lanes: 1, roots; 2, stems; 3, leaves; 4, flowers; and 5, siliques. mRNA was isolated from 25-day old light-grown plants. (II) Expression of the same genes in etiolated seedlings treated with various inducers. Lanes: 1, control; 2, wounding; 3, anaerobiosis; 4, LiCl; 5, indoleacetic acid; and 6, cycloheximide. mRNA was extracted from 5-day-old dark-grown seedlings. Each lane contains 3 μ g poly(A)⁺RNA. From Liang *et al.* (1992). The authors used the designation ACC for ACC synthase genes.

The transcription of primary response genes is usually kept repressed by a rapidly cycling repressor and they usually encode regulatory proteins/enzymes with a short half-life.

ACC oxidase is also encoded by a multigene family with several members in any one plant. These genes are also differentially regulated. For instance, in tomato, as shown by gene-specific probes, three ACO genes show distinct patterns of expression. *ACO1* is highly expressed in wounded or senescing leaves, in senescing flowers, and in ripening fruit; *ACO2* expression is not detectable either in leaves or in fruit, whereas *ACO3* is expressed in senescing leaves and flowers, but is expressed very little and transiently in fruit (Table 11-3).

5. ETHYLENE SYNTHESIS MUTANTS

Synthesis mutants for ethylene, with a reduced production of ethylene, have not been found. It is not clear whether they have not been found because of multiple isoforms of ACC synthase or ACC oxidase in a plant or because the phenotypes of an ethylene-deficient mutant and the normal wild-type plant would be indistinguishable. In contrast, ethylene-overproducing mutants are easy to identify because of thick swollen stems or petioles or by the "triple response"—tight hooks, swelling, and reduced elongation of the hypocotyl or epicotyl—in dark-grown seedlings.

Several ethylene-overproducing mutants are known in *Arabidopsis* (e.g., *eto1*, *eto2*, *eto3*) (see also Chapter

21). These mutants overproduce ethylene in the seedling stage (not as adult plants) and can be restored to the wild type by the application of AVG or AIB, which inhibit ACC synthase and ACC oxidase activities, respectively (see Table 11-2). One of these mutants, *eto2*, is now known to overproduce ethylene because it is defective in the regulatory control of one of the ACS genes (*ACS5*). This gene is specifically expressed in the seedling stage and is induced by low concentrations of cytokinins.

Other mutants show the same phenotype as ethylene-overproducing mutants, i.e., stem and petiolar swelling, epinasty, abundant roots, and compact growth habit, but where the mutant phenotype is not restored to wild type by treatment with ethylene biosynthesis inhibitors (e.g., *ctrl* in *Arabidopsis*, *epi* in tomato). These mutations occur in the response pathway of ethylene and are covered in Chapter 21.

6. REGULATION OF ETHYLENE LEVELS IN THE PLANT

6.1. Regulation of Ethylene Synthesis

As mentioned earlier, ethylene is produced at low basal rates throughout plant development, but its production is sharply increased during seed germination, ripening of climacteric fruits, and senescence and abscission. Treatment with auxins also causes enhanced ethylene production, as do stresses of various kinds. Such elevation can be provided by increased activity of three enzymes: SAM (or AdoMet) synthase, ACC synthase, and ACC oxidase. SAM synthase is the enzyme catalyzing the linkage of methionine and ATP to form *S*-adenosylmethionine (SAM) (see Fig. 11-3). SAM is the universal donor of the methyl group for DNA or protein transmethylation reactions, which are catalyzed by various methyl transferases (for DNA methylation, see Chapter 4). SAM is also the donor of the aminopropyl group for ACC production and for the synthesis of polyamines (see Box 11-2). However, the supply of SAM is usually not the rate-limiting step in ethylene biosynthesis.

In almost all cases of a sharp rise in ethylene production, the endogenous content of ACC rises from basal low levels to high levels, and there is an increase in ACC synthase activity. Hence, it is generally accepted that ethylene synthesis is regulated at the level of ACC synthase. In contrast, ACC oxidase has been thought to be constitutively expressed in most vegetative tissues, although its synthesis is enhanced during fruit ripening, senescence, and under stress conditions.

TABLE 11-3 Expression of *ACO1*, *ACO2*, and *ACO3* Genes in Tomato^a

Gene	Leaves ^b			Flowers ^c			Fruit ^d			
	UW	W	OS	(2)	(3)	(4)	MG	BK	+3	+8
<i>ACO1</i>	1	11	27	10	16	54	3	38	108	62
<i>ACO2</i>	nd ^e	nd	nd	10	23	25	nd	nd	nd	nd
<i>ACO3</i>	nd	nd	13	23	58	96	nd	3	1	nd

^aThe relative abundance of ACO transcript levels was quantified using a radioanalytical imaging system. Reprinted with permission from Barry *et al.* (1996), © Blackwell Science Ltd.

^bUW, unwounded leaves; W, leaves after wounding for 2 h; and OS, leaves at the onset of senescence.

^cWhole flowers harvested prior to anthesis (2), at anthesis (3), and at the onset of senescence (4).

^dFruit harvested at the mature green (MG) stage, the first visible sign of color change, i.e., breaker stage (BK), and 3 (+3) and 8 (+8) days after the onset of color change.

^end, transcript not detectable.

6.2. Ethylene Promotes as well as Inhibits Its Own Production

Depending on the tissue, ethylene can autocatalyze or autoinhibit its own production. If green, but mature, tomato fruits are treated with ethylene, there is a dramatic increase in the transcripts of both ACC synthase and ACC oxidase genes. Inhibitors of ethylene action, such as 2,5-norbornadiene, block the accumulation of these transcripts. These data suggest that, in normal fruit ripening, the low basal levels of ethylene present in green fruits trigger the massive production of ethylene by enhancing the synthesis of ACC synthase and ACC oxidase (Fig. 11-7). Similar data are known for presenescent carnation petals. Ethylene can also inhibit its own production. For example, slicing of citrus flavedo tissue causes wound ethylene production, which is inhibited greatly by exogenous ethylene. In one experiment, ethylene production in the cut tissue increased more than 20-fold, whereas in the cut tissue supplied with exogenous ethylene, there was little change.

How can the same signal, i.e., ethylene, promote and inhibit its own production? The answer lies in the multiplicity of genes encoding ACC synthase and the different induction kinetics for different ACS genes. In

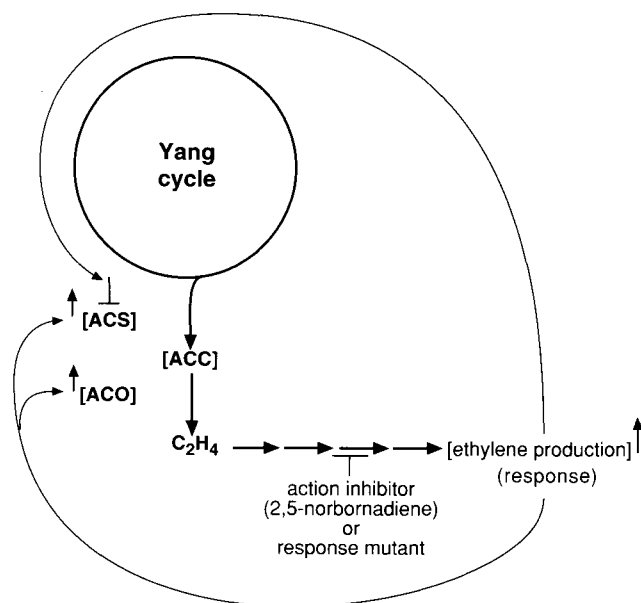


FIGURE 11-7 A schematic illustration of autocatalysis and auto-inhibition of ethylene production. Ethylene produced *via* ACC induces more ethylene production by stimulating the expression/activity of ACS and ACO enzymes. Such autocatalysis, as well as ethylene-induced responses, is inhibited by inhibitors of ethylene action, such as 2,5-norbornadiene, or in response mutants. Ethylene also inhibits its own production by inhibiting ACS expression/activity by a mechanism that is still unknown.

a study in tomato, the expression of five ACS and two ACO genes was followed during fruit development. Results showed that ACS genes fall into three groups. Group 1 genes (*LeACS1A* and *LeACS3*) are constitutively expressed at low levels throughout fruit development; group 2 genes (*LeACS2* and *LeACS4*) are not expressed in immature and preclimacteric green fruit, but their expression is sharply upregulated during fruit maturation and ripening; and the single group 3 gene (*LeACS6*) is expressed in immature and preclimacteric green fruit, but its expression is downregulated before climacteric and fruit maturation. In parallel experiments, maturing fruits were treated with MCP, a suppresser of ethylene action (see Section 8). Suppression of ethylene action by MCP had no effect on the expression of group 1 genes, the expression of group 2 genes was severely, although transiently, curtailed, and the expression of the group 3 gene, which was not expressed during maturation, was restored. The conclusions were clear. In tomato, some ACS genes are expressed constitutively and their expression is unaffected by ambient ethylene concentration (group 1 genes). These genes probably contribute to the low basal levels of ethylene production. The

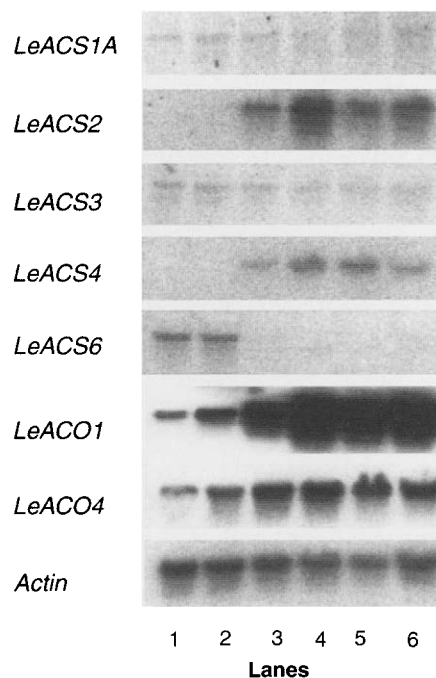


FIGURE 11-8 Expression of *LeACS* and *LeACO* genes in tomato fruit development and ripening. mRNAs were prepared from fruit at stages shown; 3 μ g was loaded per lane and hybridized to gene-specific probes. Actin was used as an internal control to normalize the amount of mRNA loaded. Fruit stages: lane 1, immature fruit; lane 2, mature green stage; lane 3, turning stage; lane 4, pink stage; lane 5, red stage; and lane 6, fully ripe stage. Modified from Nakatsuka *et al.* (1998). The authors use the designation LE-ACS2, LE-ACS3, and so on.

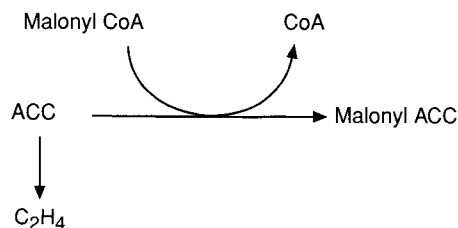


FIGURE 11-9 Formation of *N*-malonyl ACC. ACC may be converted to ethylene by ACC oxidase or to *N*-malonyl ACC by *N*-malonyltransferase. The latter pathway acts to downregulate ethylene production.

expression of other ACS genes is modulated by ethylene during fruit development. Some of these are downregulated by higher concentrations of ethylene, but can be reinduced if ethylene action is suppressed (group 3 gene), whereas others are induced by ethylene and their expression is downregulated if ethylene action is suppressed (group 2 genes). Group 2 genes contribute to the massive burst of ethylene production seen during climacteric.

ACO genes (*LeACO1* and *LeACO4*) are expressed moderately during low-level ethylene production, but their expression is sharply upregulated during fruit maturation and ripening. Judging from the effect of MCP treatment, this increased expression is ethylene induced (Fig. 11-8).

6.3. Conjugation of ACC

Ethylene synthesis can also be regulated at the level of its precursor, ACC. ACC may be oxidatively cleaved to produce ethylene or it may be conjugated with malonyl-CoA to give rise to *N*-malonyl ACC (MACC) (Fig. 11-9). The enzyme ACC *N*-malonyltransferase has been purified. Earlier it was thought that MACC was formed as a storage product and acted as a reservoir for ACC, which could be retrieved and converted to ethylene when needed. More recent work suggests that the pathway to MACC is irreversible and that this is a way to deplete ACC levels and down-regulate ethylene production.

6.4. Oxidation of Ethylene

Ethylene is oxidized to ethylene oxide and CO_2 , and the oxide can be converted to ethylene glycol, which can be further conjugated to glucose (Fig. 11-10). The actual pathways of metabolism vary in different tissues. The physiological significance of this metabolism remains to be established, however, because ethylene is a gas and can easily diffuse out.

7. SYNTHETIC COMPOUNDS THAT PRODUCE ETHYLENE

Ethephon (2-chloroethyl phosphonic acid, see Fig. 11-11) is often used to generate ethylene. It is stored at an acidic pH, but decomposes to release ethylene at physiological pH. The precursor of ethylene, ACC, can also be supplied to plants in liquid media. It is picked up and generates ethylene in plant tissues without the disturbing effects of pH that accompany the use of ethephon.

8. INHIBITORS OF ETHYLENE ACTION

Because many fruits produce ethylene, which in turn makes them ripen, it is of interest to fruit growers and shippers to regulate the production or amount of ethylene in the vicinity of fruits. Similarly, ethylene production causes fading of color and senescence and abscission of cut or potted flowers, and regulation of ethylene levels is of interest to the flower industry. Several inorganic and organic compounds are known to either scavenge ethylene from atmosphere and thus lower its concentration or inhibit ethylene action. For instance, potassium permanganate ($KMnO_4$) can reduce the concentration of ethylene in apple storage areas from 250 to $10 \mu l \cdot liter^{-1}$ and, thus, extend the storage life of the fruit.

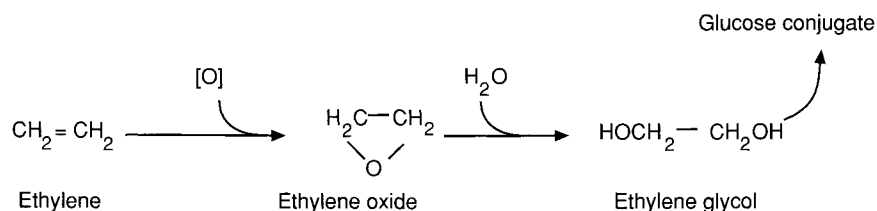


FIGURE 11-10 Oxidative breakdown of ethylene.

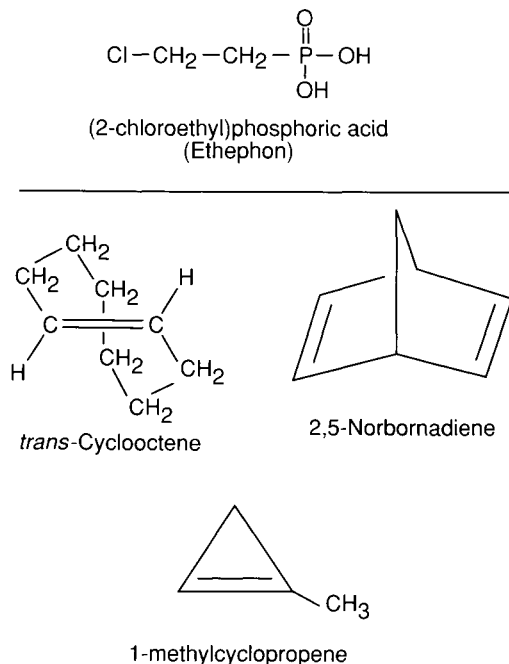


FIGURE 11-11 Structure of Ethephon and some inhibitors of ethylene action: 2,5-norbornadiene, *trans*-cyclooctene, and 1-methylcyclopropene.

Silver salts, especially silver thiosulfate, and organic compounds such as 2,5-norbornadiene, *trans*-cyclooctene (but not the *cis* form), other cyclic olefins, and *cis*-butene inhibit ethylene action (Fig. 11-11). These compounds have been used in agriculture and horticulture industries to delay or prevent fruit ripening or the senescence and abscission of flowers for a long time. Their mechanism of action is not understood (see Chapter 21), but there are several disadvantages to their use. Metal ions, and norbornadiene and *trans*-cyclooctene, while inhibiting ethylene action, are also known to induce ethylene synthesis by plant tissues. Silver is a heavy metal, and its use in food and cattle feed industries is not acceptable. 2,5-Norbornadiene and *trans*-cyclooctene have a foul odor and need to be used in large concentrations.

For these reasons, in recent years, some other compounds, e.g., cyclopropene (CP), 1-methylcyclopropene (1-MCP), and 3,3-dimethylcyclopropene (3,3-diMCP), have been developed that inhibit ethylene action in a competitive manner. These compounds, especially 1-MCP, are effective at relatively low concentrations (Fig. 11-12) and, because they are odorless gases, can be used for large samples in a closed environment. It has been reported that their effect persists for a long duration (2–3 weeks or longer at cooler temperatures), but in a study on ACS and ACO gene



FIGURE 11-12 Carnation flowers. The photograph was taken 7 days after the flower on the right had been treated for 6 h with 200 ppb ($0.5 \text{ nl} \cdot \text{liter}^{-1}$) 1-MCP. Untreated flower on the left. Courtesy of Michael Reid, University of California, Davis.

expression in tomato, the effect was transient (see Section 6.2).

9. PRODUCTION OF TRANSGENIC PLANTS

An understanding of the steps in ethylene biosynthesis and cloning of genes of the enzymes, ACC synthase and ACC oxidase, has allowed the production of transgenic plants with altered levels of ethylene. Transgenic tomatoes have been produced that have low levels of ethylene and thus have fruits that mature but fail to ripen or ripen very slowly. These fruits can have a shelf life of 6 months or more and can be ripened on demand by exposure to ethylene. For tomatoes, this has been accomplished in several ways (Fig. 11-13). The introduction of ACS or ACO cDNA in an antisense orientation silences the expression of the corresponding endogenous genes and results in plants that produce very low levels of ethylene. Introduction of a bacterial gene from *Pseudomonas* for ACC deaminase results in deamination of ACC such that it is no longer available for ethylene biosynthesis. Similarly, the introduction of a gene encoding *S*-adenosyl-methionine hydrolase removes AdoMet from the intracellular pool, leaving no substrate for ACC synthase to act upon.

10. CHAPTER SUMMARY

Ethylene, a simple organic molecule, has important roles in the ripening of many fruits, in the induction

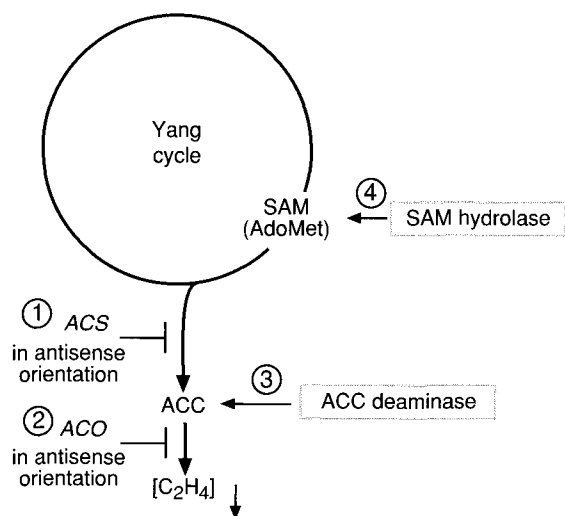


FIGURE 11-13 Four ways to reduce ethylene production by plants: (1) silencing of ACS gene expression, (2) silencing of ACO gene expression, (3) deamination of ACC, and (4) hydrolysis of S-adenosylmethionine.

of senescence in leaves and flowers, and in the abscission of leaf petioles and flower peduncles. It also maintains the apical hook during the germination of dicot seeds and acts to trigger defense responses under stressful situations, such as flooding, extremes of temperature, wounding, and pest and pathogen attack. Ethylene is synthesized in flowering plants from methionine *via* a cyclic pathway, which preserves the

methylthio group of methionine while using ATP. Two notable intermediates in the cycle are S-adenosylmethionine and 1-aminocyclopropane-1-carboxylic acid; the latter compound is the immediate precursor of ethylene. The enzyme, ACC synthase (ACS), cleaves SAM to yield ACC and is the key regulatory enzyme in ethylene biosynthesis. The second enzyme ACC oxidase (ACO) cleaves ACC to yield ethylene. These enzymes are encoded by multigene families in all plants investigated, and different members of these gene families are expressed differentially in different tissues and organs in response to developmental cues, as well as environmental factors and hormones, such as auxins, cytokinins, and ethylene itself. Ethylene also acts to inhibit its own production. While synthesis mutants of ethylene have not been found, several mutants in *Arabidopsis* overproduce ethylene; one of them does so because the regulatory control over one of the ACS genes is lost. Inhibitors of ethylene production inhibit the activity of either of the two enzymes, ACC synthase or ACC oxidase. Inhibitors of ethylene action are also known and include silver ions as well as many organic compounds, such as 2,5-norbornadiene, *trans*-cyclooctene, and 1-methylcyclopropene. These compounds are of importance in fruit and flower industries because they can prevent or delay overripening of harvested fruit or flower senescence and abscission while in storage or in shipment. Transgenic silencing of ACC synthase or ACC oxidase genes has also been accomplished and used to delay ripening of fruits.

BOX 11-2 POLYAMINES: SYNTHESIS AND POSSIBLE PHYSIOLOGICAL ROLES¹

POLYAMINES (PAs) ARE LOW molecular weight, strongly basic molecules found ubiquitously in all living organisms, bacteria, fungi, animals, and plants studied so far. In plant cells, diamine putrescine, triamine spermidine, and tetraamine spermine constitute the major PAs.

Chemical structures:

Putrescine $\text{H}_2\text{N}-(\text{CH}_2)_4-\text{NH}_2$

Spermidine $\text{H}_2\text{N}-(\text{CH}_2)_3-\text{NH}-(\text{CH}_2)_4-\text{NH}_2$

Spermine $\text{H}_2\text{N}-(\text{CH}_2)_3-\text{NH}-(\text{CH}_2)_4-\text{NH}-(\text{CH}_2)_3-\text{NH}_2$

They occur mainly in the free form or bound to phenolic acids, other low molecular weight compounds, or macromolecules such as proteins and nucleic acids. At physiological pH, all PAs are positively charged and have been shown, *in vitro*, to bind strongly to negatively charged nucleic acids, acidic phospholipids, and many types of proteins, including enzymes whose activities are directly modulated by PA binding. Through such binding, PAs are known to affect the synthesis and activity of macromolecules and processes of mitosis and meiosis.

¹ Box 11-2 text courtesy of Ravindar Kaur-Sawhney, Department of Biology, Yale University, New Haven, CT.

PAs are synthesized in plants in two major steps. Putrescine is synthesized from arginine or ornithine via arginine decarboxylase (ADC) or ornithine decarboxylase (ODC), respectively (Fig. 11-14). Spermidine and spermine are synthesized from putrescine by successive transfers of aminopropyl groups from S-adenosylmethionine (SAM). For such a transfer, SAM is first decarboxylated by SAM decarboxylase, and the aminopropyl groups are attached to spermidine or spermine via specific synthetases. As explained earlier, SAM is also a precursor for ethylene biosynthesis via ACC. ADC and ODC can be inhibited irreversibly by DL- α -difluoromethylarginine (DFMA) and DL- α -difluoromethylornithine (DFMO), respectively. Methylglyoxal-*bis*-guanyl hydrazone (MGBG) and cyclohexylamine are reversible inhibitors of SAM decarboxylase and spermidine (or spermine) synthetase activities, respectively.

The growth-promoting activities of PAs were first shown in bacteria and yeast in the 1950s. Research was then extended to animals and to plants. In some bacteria, mutants lacking the ability to synthesize PAs are unable to grow and develop normally. Because the addition of PAs to these mutants generally restores normal growth and development, it is reasonable to conclude that PAs are essential to these cells. This conclusion is reinforced by the demonstrable effect of the "suicide inhibitors" DFMA and DFMO, which specifically and irreversibly bind to ADC and ODC, respectively. The ensuing decline in cellular PA titers is accompanied by a decrease or cessation of growth and development, which are restored by the addition of the relevant PA.

In plants, PAs are known to be involved in many growth and developmental processes, such as flowering, cell division, macromolecular synthesis, stress response, and senescence, and have been tentatively proposed as a new class of plant growth substances.

Flower induction and development: Free and conjugated PAs accumulate in the shoot apex, buds, and flowering parts of many plants. In *Arabidopsis*, spermidine concentrations are low in rosettes and bolts but increase dramatically in flowers. Tobacco thin-layer cultures can be programmed to produce flowers or vegetative buds. Cultures programmed to flower show high endogenous levels of spermidine. If these cultures are treated with increasing concentrations of cyclohexylamine, a reversible inhibitor of spermidine synthetase, the cultures progressively revert to the vegetative state as the spermidine concentration decreases. The addition of spermidine reverses inhibition and increases flowering. In contrast, cultures programmed to produce vegetative buds show a low concentration of endogenous spermidine, but can be induced to produce flower buds with exogenous applications of spermidine (Fig. 11-15). These results

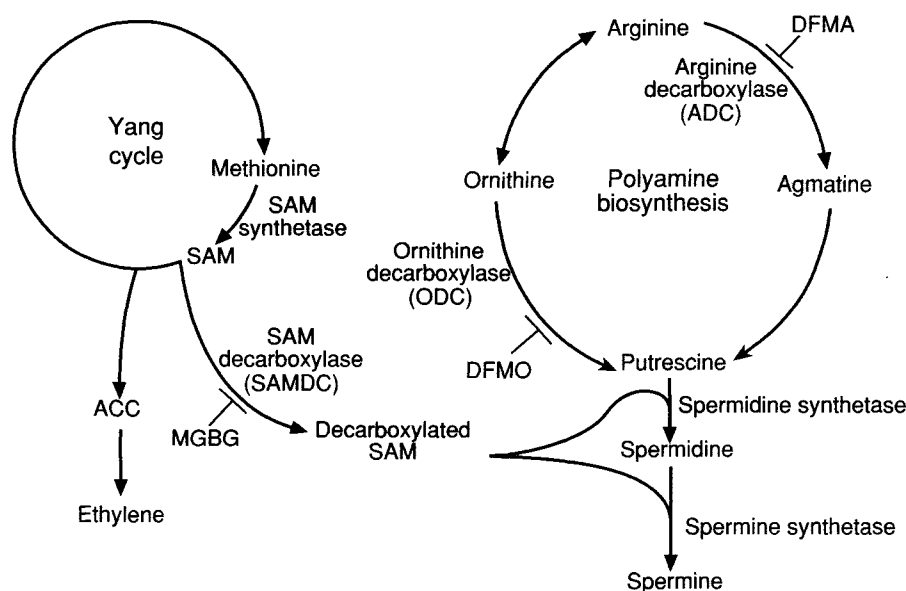


FIGURE 11-14 Polyamine and ethylene biosynthesis pathways and their interrelationships. Inhibitors of enzymes in polyamine biosynthesis are indicated. SAM, S-adenosylmethionine; ACC, 1-aminocyclopropane-1-carboxylic acid; MGBG, methylglyoxal-*bis*-guanyl hydrazone; DFMA, DL- α -difluoromethylarginine; DFMO, DL- α -difluoromethylornithine.



FIGURE 11-15 Effect of spermidine on bud differentiation in thin-layer explants of tobacco. Explants were cultured in the presence (right) or absence (left) of 5 mM spermidine. Photographs were taken when the cultures were 1 month old and buds were fully developed (X 2.5). From Kaur-Sawhney *et al.* (1988).

with *in vitro* cultures are supported by stronger correlations between endogenous PA concentration and flowering in whole plants. In many short and long-day plants, photoperiodic induction causes an increase in endogenous PAs as a prelude to floral initiation. Inhibition of this increased PA biosynthesis by DFMO, and sometimes by DFMA, prevents both PA increase and flowering. In *Pharbitis nil*, a short-day plant that can be induced to flower by a single long night, putrescine is effective in inducing flowering under noninductive conditions.

Polyamines and plant hormones: Several reports suggest an interaction between PAs and plant hormones. Most studies have examined changes in endogenous PA content and biosynthesis as a result of hormone treatment and have correlated these changes with growth. The effects of auxins on PA metabolism were first reported in the tubers of Jerusalem artichoke (*Helianthus tuberosus*). In dormant tubers, which contain low PA titers, cell division could be stimulated by treatment with exogenous PAs or IAA. Because auxin application caused a large increase in PA content, it was suggested that auxins act through PAs to promote growth in this tissue. Similarly, IBA-induced root formation on mung bean hypocotyl cuttings was accompanied by a 2- and 1.5-fold increase in putrescine and spermidine content, respectively. Inhibition of this increase by PA synthesis inhibitors decreased root formation. The decrease was reversed by the exogenous application of arginine or ornithine, suggesting that PAs are necessary for IBA-induced root formation. Several other studies have reported an increase in ODC or ADC activities following auxin treatment. In tomato, auxin (NAA) treatment of unfertilized ovaries can cause fruit set and growth (parthenocarpy, see Chapter 17). This parthenocarpic growth is inhibited by DFMO, and putrescine reverses the DFMO effect. Thus it appears that auxin-mediated PA biosynthesis is required for tomato ovary development. In a few instances, however, auxins have been shown to inhibit PA biosynthesis and cause a decrease in PA titer. The reasons for these discrepancies are not clear.

One of the bioassays for cytokinins is the promotion of cotyledonary expansion in dicot seedlings. Cucumber seedlings supplied with cytokinins not only show cotyledonary expansion, but also an increase in PA biosynthesis and titer. Moreover, cytokinins can reverse an inhibitory effect of abscisic acid on PA biosynthesis. In the buds of etiolated pea seedlings, where red light promotes growth and ADC activity, cytokinins further augment the activity of ADC. Suspension cultures of plant cells usually require both a cytokinin and an auxin for cell division and growth. Such growth stimulation in suspension cultures of

rice could be mimicked by spermidine, although not by putrescine or spermine. Aging of pea leaves is accompanied by a decrease in PA levels. Application of kinetin delayed senescence and reduced the decrease in PA titer.

Similar to data with auxin or cytokinin applications, GA treatments are also followed by an increase in PA levels and the activities of their biosynthetic enzymes. The GA-induced elongation of the dwarf pea internode in light is accompanied by a rise in ADC activity and PA titer, whereas the application of DFMA partially prevents these increases. This elongation involves both cell division and cell elongation; PAs appear to play a role in cell division but not in cell elongation. The GA-induced α -amylase production in barley aleurone layers is accompanied by PA biosynthesis, and PAs, particularly spermidine, are thought to be involved in GA-dependent amylase production.

Thus, it appears that PA metabolism is affected by auxins, cytokinins, and gibberellins in several plant systems and that PAs are essential for many of the growth responses attributed to these hormones. The specific roles of PAs in these responses are unknown, but it is unlikely that the entire spectrum of hormonal effects in these responses is mediated directly by changes in PA metabolism.

PAs and ethylene play antagonistic roles in plant responses, such as ripening of climacteric fruits and senescence of leaves and flowers. Ethylene promotes both responses, whereas PAs inhibit them. As mentioned earlier, PAs and ethylene are synthesized from a common precursor, SAM, by two separate pathways. The potential for competition for SAM between the two pathways, especially when demands for ethylene or polyamines are high, is well recognized. Also, ethylene is an effective inhibitor of SAM decarboxylase and ADC, key enzymes in the PA biosynthetic pathway. In contrast, PAs are known to inhibit ethylene biosynthesis, perhaps by blocking the conversion of SAM to ACC and of ACC to ethylene. Thus, it has been proposed that some effects of PA on senescence and fruit ripening are due to a restriction of ethylene biosynthesis.

In summary, PAs are generally recognized as active regulators of plant growth. They are present in all cells, and their millimolar titer is responsive to physiological effects caused by many agents, such as hormones, light, and stress, but their precise mode of action in plant growth and development is still unclear. Molecular genetic techniques are beginning to be used for the analysis of PA biosynthesis, as well as their interaction with hormones. Genes for SAM decarboxylase have been cloned and others are likely to follow.

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Jasmonates and Other Defense-Related Compounds

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1. INTRODUCTION

In addition to the hormones considered so far, there are some other naturally occurring substances that have growth-, stress-, or defense-related activities. Among these, jasmonates, a class of compounds known as oxylipins with roles in plant growth regulation and defense, have a wide distribution in plants, are active at small concentrations, occur naturally in small amounts, and have pleiotropic effects. Most authors now regard them as plant hormones. Other substances include oligosaccharins, which are short oligosaccharide fragments from plant or fungal cell walls; systemin, an 18 amino acid polypeptide; and

salicylic acid. These compounds have limited occurrence and seem more to be part of signal transduction pathways related to defense. The list of naturally occurring substances with growth- or defense-related activities is by no means complete, and more substances may be added in future years. This chapter deals with jasmonates. A short account of plant defense responses is provided in Box 12-1, and the role of systemin in wound signaling is highlighted.

2. DISCOVERY, DISTRIBUTION, AND STRUCTURE OF JASMONATES

Jasmonic acid (JA) and its fragrant methyl ester, methyl jasmonate (MeJA), a volatile constituent of the essential oil of jasmine, rosemary, and many other flowers, became of interest to plant physiologists in the early 1980s when they were shown to retard the growth of roots and coleoptiles and to promote leaf senescence. These activities immediately led to their being tested in many other bioassays. In more recent years, they have come to be regarded as being the major inducers of plant defense against insect and pathogen attack, and stress- and senescence-related responses. Both JA and MeJA are biologically active and, together with their derivatives, are referred to as jasmonates.

Jasmonates are common in plants; they have been recorded by radioimmunoassay in more than 160 families of angiosperms and gymnosperms; they also occur in green and red algae and in several fungi.

The chemical structure of JA is shown in Fig. 12-1. The JA molecule has two chiral centers at C-3 and C-7. Because each chiral center can have an *R* or *S* absolute configuration, there are four possible stereoisomers. The mirror image isomers, (3*R*, 7*S*) and (3*S*, 7*R*), also known as (+)-7-iso-JA and (–)-7-iso-JA, have both their side chains in *cis* orientation with respect to the plane of the cyclopentanone ring. The other pair, (3*R*, 7*R*) and (3*S*, 7*S*), or (–)-JA and (+)-JA, have their side chains in a *trans* configuration. Because of increased steric hindrance, the *cis* configuration is less stable and epimerizes to the *trans* configuration.

The principal naturally occurring jasmonates in plants are the (–)-JA (compound I in Fig. 12-1) and its methylated derivative, methyl jasmonate (V). Smaller amounts of (+)-7-iso-JA (II) are also found, but the other two isomers (III and IV) occur mainly

in commercial preparations and have little biological activity. Commercially available jasmonates are prepared as methyl jasmonates and are a mixture of (+/–)-MeJA:(+/-)-7-iso-MeJA in a ~ 9:1 ratio.

3. PHYSIOLOGICAL ROLES OF JASMONATES

Although JA seems to be more prevalent in plant materials, MeJA is the more active compound when applied exogenously, probably because it is volatile and lipophilic. JA and MeJA serve important functions in plant defense responses against mechanical wounding or wounding by herbivores and attack by pathogens. To understand this role better, a short introduction to plant defense responses is provided in Box 12-1.

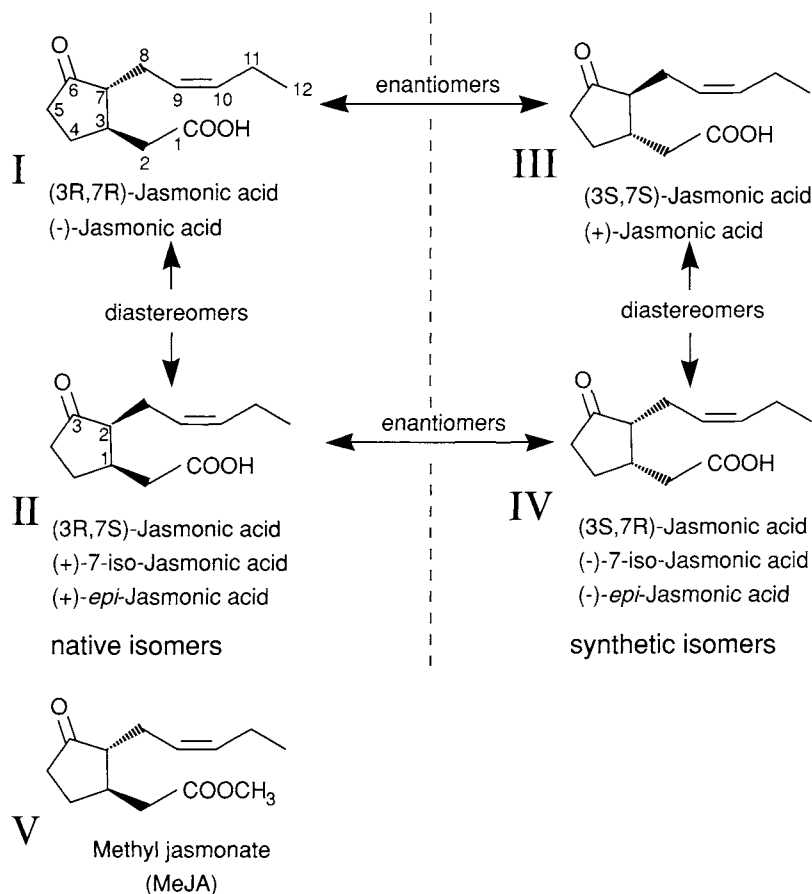


FIGURE 12-1 Structures of stereoisomers of jasmonic acid [3-oxo-2-(2'-*cis*-pentenyl)-cyclopentane-1-acetic acid. *Note:* This designation of JA follows the old carbon numbering system for the ring shown partly in compound II. The -7-iso- is synonymous with the -2-*epi*-designation.] The structure of methyl jasmonate (MeJA) is also shown. Modified from Sembdner and Parthier (1993).

BOX 12-1 PLANT DEFENSE RESPONSES

PLANTS HAVE EVOLVED AN elegant armory of defense systems against attack by insect herbivores and microbial pathogens. They synthesize a wide variety of compounds, including wall substances, toxic proteins and peptides, enzymes, and secondary metabolites. Some are constitutively produced, whereas others are induced by wounding or microbial attack. Among wall substances, the synthesis of lignin at a wound site and its deposition in cell walls, thus strengthening them, is part of defense strategy. Lectins are glycoproteins that recognize specific sugar residues in carbohydrates on the intestinal tract of insect or mammalian herbivores or bacterial or fungal walls. Several hundred lectins have been described in plants. Because most have multiple sugar-binding domains, they are capable of binding several oligosaccharides at the same time and, thus, agglutinating animal cells (e.g., red blood cells) or precipitating glycoconjugates. Ribosome-inactivating proteins (RIPs) also bind to carbohydrates, but act as N-glycosidases, which specifically remove adenine residues from polynucleotides, such as DNA and RNA. Lectins and RIPs occur not only in seeds, but also in vegetative organs, such as bark, leaves, storage bulbs (for additional details, see Chapter 20).

Other defense chemicals include enzymes and products, which are harmful to invading organisms. For example, proteinase inhibitors (PINs) in potato or tomato shoots, when ingested by the insect, inhibit the protein-digesting enzymes, trypsin and chymotrypsin, in the insect gut. α -Amylase inhibitors inhibit the activity of the starch-digesting enzyme, α -amylase. Patatin, the most abundant protein in potato tuber, is a nonspecific acyl hydrolase, which hydrolyzes a variety of lipids—phospholipids, glycolipids, and mono- and diacylglycerols, all except triacylglycerols. Many pathogenesis-related (PR) proteins are synthesized as defense against microbial pathogens. For example, chitinases and β -1, 3-glucanases provide defense by digesting the cell walls of invading bacteria and fungi. Phytoalexins are a chemically heterogeneous group of secondary metabolites that accumulate around the site of infection and are believed to be toxic to the invading pathogen. Different plant families employ different types of secondary products as phytoalexins. For example, isoflavonoids are common phytoalexins in the legume family, whereas in plants of the potato family (Solanaceae), various sesquiterpenes are produced as phytoalexins.

Defense compounds also include a variety of volatiles, which are released in the atmosphere. The volatiles are products of different metabolic pathways: indole compounds derived via the shikimic acid pathway, mono- and sesquiterpenes derived from the isoprenoid pathway, various aldehydes, and jasmonates derived via the lipoxygenase (LOX) pathway (Fig. 12-2, see also Figs. 6-2 and 7-5 in Chapters 6 and 7, respectively). Some of these volatiles act as potent defense chemicals against feeding insects and microbial pathogens by either repelling them or being toxic to them. Some act as signaling molecules, which attract natural enemies, parasites and predators, of plant-feeding insects, e.g., the parasitoid wasp, which lays its eggs in the gut of the feeding caterpillar.

These chemicals may be part of defense systems that are activated constitutively (e.g., many lectins, RIPs, glycosides) or they may be part of inducible systems that are activated upon feeding by herbivores or pathogenic attack (e.g., lignin, some lectins, PINs, PR proteins, phytoalexins, and some volatiles). Some of the important enzymes in the phenylpropanoid pathway leading to lignin that are induced include phenylalanine ammonia lyase (PAL) and chalcone synthase (CS). Inducible defense responses require recognition by plant cells of a signal from the invading organism (see later). This recognition event triggers a signaling cascade leading to, among other things, gene transcription and new protein synthesis. Defense-related proteins, mostly enzymes or their products, are generally undetectable in a plant before, but accumulate rapidly after attack. In comparison to constitutive systems, inducible systems provide a relatively economical defense because they do not require a permanent outlay of plants' resources. Hence, they are more common in vegetative parts, especially leaves.

The subject of plant defense is vast. Several hormones and growth regulators are involved in these responses, including jasmonates, ABA, ethylene, and salicylic acid. Among these jasmonates and ABA seem to be the principal hormones in defense against wounding (including herbivores), whereas jasmonates, ethylene, and salicylic acid seem to be involved in the defense against phytopathogens. ABA and

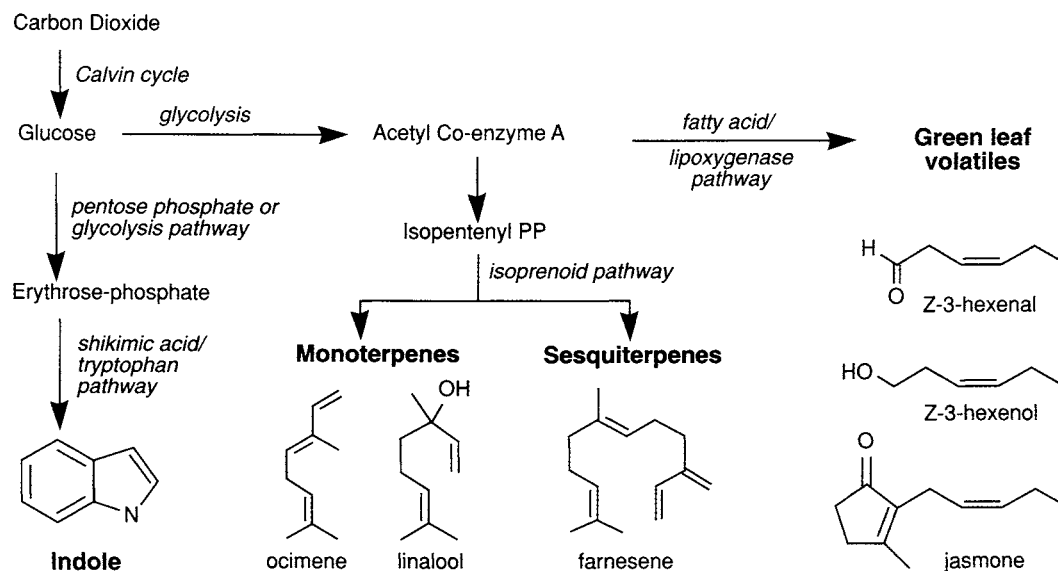


FIGURE 12-2 Biosynthetic routes leading to three classes of volatiles—indole, terpenoids, and green leaf—released from cotton plants by insect feeding on the leaves. Isopentenyl diphosphate, shown here as derived from acetyl-CoA, could also be derived from plastidic sources (see Fig. 7-6 in Chapter 7). Modified from Paré and Tumlinson (1997).

jasmonates seem to be involved in defense against abiotic stresses. Many of the defense-related proteins, proteinase inhibitors, some PR proteins, and volatiles are either induced by jasmonates or synthesized by alternate pathways in jasmonate biosynthesis.

3.1. Jasmonates Induce Defense Responses

Mechanical wounding or chewing by insect herbivores results in the production of an 18 amino acid polypeptide called systemin. Systemin itself is the product of proteolytic cleavage of a much larger protein called prosystemin. The gene encoding prosystemin is transcriptionally activated on wounding. Such activation occurs mostly in vascular cells, and systemin is probably cleaved *in situ*. It is transported long distances in phloem tissue and causes defense-related proteins to be synthesized in leaves close to and far away from the wound site. Wounding or attack by microbial pathogens may also produce oligosaccharide fragments from the plant or fungal cell walls. "Elicitors" are compounds of microbial origin; they can be proteins, peptides, lipids, and polysaccharides, including wall fragments, that are recognized by the host plant and initiate the defense responses. Oligosaccharide fragments released from plant cell walls can also act as elicitors. Although elicitors or oligosaccharides are not transported long distance in phloem tissue, they may nonetheless induce the expression of the prosystemin gene and thereby the synthesis of systemin. Wounding or treatment of plant tissues or cells in culture with

systemin or oligosaccharide fragments induces the synthesis of JA and/or MeJA, which, in turn, brings about the expression of defense-related genes (Fig. 12-3). Such induction of JA/MeJA by systemin has been reported so far only in members of Solanaceae, such as tomato (*Lycopersicon* sp.). Methyl jasmonate, being volatile, can be airborne from an injured to a healthy plant and has been suggested to play a role in interplant communication of the distress signal. So far, such a role has been restricted to plants of the same species under greenhouse conditions. ABA is also thought to be involved in wound response because ABA synthesis mutants of potato and tomato do not show induction of response unless supplied ABA.

Table 12-1 shows the increase in endogenous levels of JA in leaves after wounding.

3.2. Other Roles

Jasmonates are also synthesized under other types of stress (e.g., water stress, see Fig. 12-5) or developmental conditions. Moreover, JA or MeJA induces its own synthesis and the synthesis of volatiles derived from the lipoxygenase (LOX) pathway (see Section 4.2).

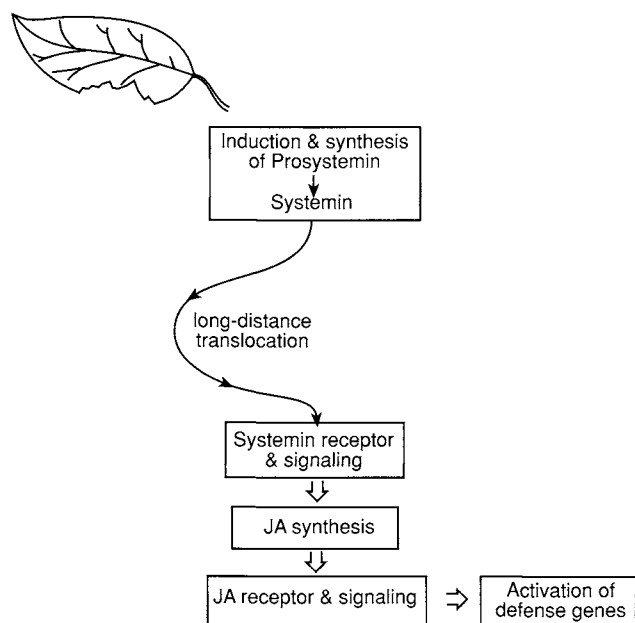


FIGURE 12-3 Proposed signaling pathway for the induction of defense-related genes following wounding in tomato (Solanaceae). The wound signal is recognized by plant cells in a still unknown manner, but it leads to the synthesis of prosystemin and its subsequent cleavage to yield systemin. Systemin is transported long distances in the phloem to tissues far from the wound site. It is recognized by its receptor, which is thought to be located on the surface of target cells. The recognition of systemin by its receptor initiates a signal transduction chain, which results in the synthesis of jasmonates. Jasmonates, in turn, induce the expression of defense-related genes, which have the effect of discouraging herbivory. Modified from Farmer and Ryan (1992).

Jasmonates are involved in the synthesis of vegetative storage proteins (VSPs) in vegetative parts of the plant. These proteins are synthesized when nutrient supply exceeds their demand for growth and, subsequently, are utilized either in seed development in annual plants or spring growth in perennial trees and shrubs. Many plants form tubers or bulbs for storage

TABLE 12-1 Increase in Endogenous Levels of JA in Leaves of *Bryonia dioica* and *Avena* Following Wounding^a

Species	JA (pmol g fw ⁻¹)		Increase (× control)
	Control	Wounded	
<i>Bryonia</i>	47 ± 12	314 ± 57	× 6.7
<i>Avena</i>	64 ± 22	218 ± 79	× 3.4

^aWounded leaves were harvested after 30 (*Avena*) or 120 (*Bryonia*) min. Results are averages of four separate experiments for each species (± SD). JA levels were measured by ELISA using monoclonal antibodies prepared against (–)-JA. From Albrecht *et al.* (1993).

of food materials (e.g., potato, onion). Jasmonates and some of their derivatives have been proposed to stimulate tuber or bulb formation. VSPs and tuberization are covered in more detail in Chapter 20.

Many climbing plants coil around a support by their tendrils. Coiling is a fascinating form of growth, although little is known about its mechanism. It can be induced, by mechanical touching of a support (a thigmotropic response) or abrasion. It is also caused by exposure of plant tissues to MeJA (Fig. 12-4) and, even more potently, by the methyl derivative of an intermediate in JA biosynthesis, 12-oxo-PDA (see Section 4).

Jasmonates share some growth-inhibiting or stress-related responses with ABA, although they probably do so by separate pathways. Like ABA, JA inhibits root and shoot growth and germination of nondormant seeds. Also, similar to ABA, JA levels rise in plant tissues under conditions of osmotic stress (Fig. 12-5), but do not result in induction of the same genes.

Jasmonates also share some responses with ethylene and are thought to promote fruit ripening, senescence of leaves and flowers, and abscission of petioles and peduncles. Fruit ripening is covered in Chapter 17, and senescence and abscission are covered in Chapter 20. Plant defense responses against phytopathogens involve jasmonates, ethylene, or salicylic acid. These responses are covered in Chapter 24 under jasmonate signaling. In summary, although jasmonates share many responses with ABA or ethylene (or salicylic acid), they seem to regulate these responses, or different components of them, independently. For example, the synthesis of VSPs and some defense- or pathogenesis-related proteins induced by jasmonates is not induced by either ABA or ethylene.

Bioassays for jasmonates are based on their ability to induce the expression of defense-related proteins, or genes encoding them, their ability to inhibit root growth, and their ability to induce tendril coiling.

Treatment of cells in culture with elicitors derived or from plant or fungal cell walls with systemin, or mechanical wounding of isolated leaves, provide valuable *in vitro* systems to study JA biosynthesis and its regulation as well as expression of JA-induced genes. This topic is covered in the next section.

4. BIOSYNTHESIS OF JASMONIC ACID (JA)

Jasmonic acid is derived from a polyunsaturated fatty acid that occurs in membrane lipids; therefore, some important aspects of fatty acid metabolism are presented in Box 12-2.

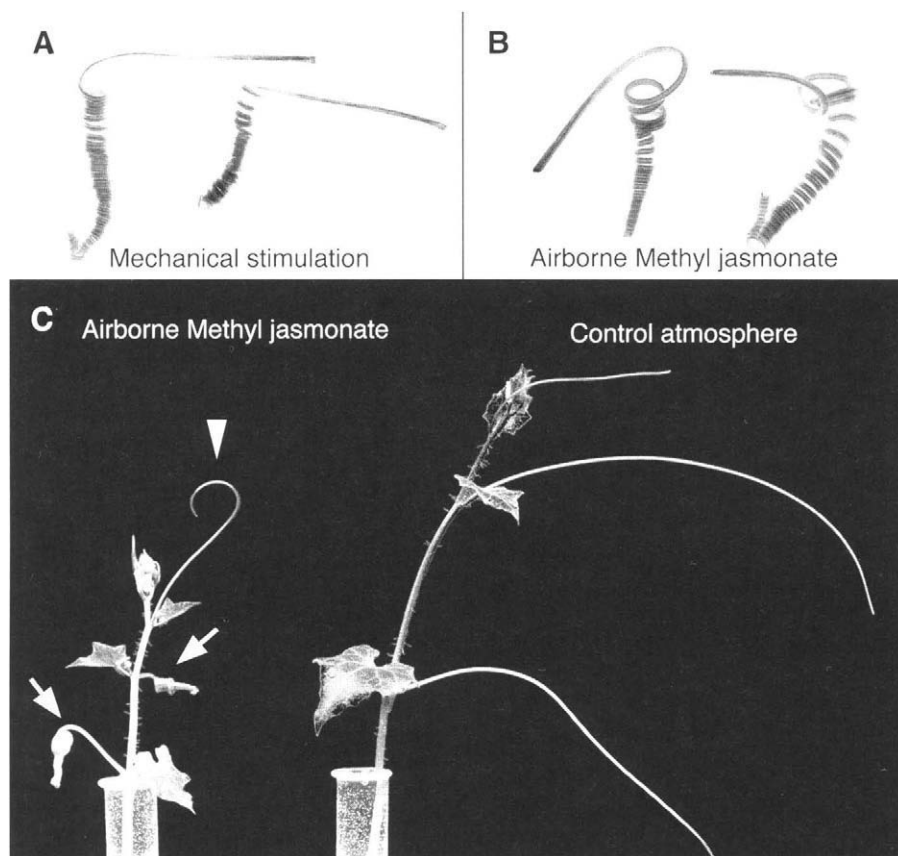


FIGURE 12-4 Tendril free coiling (i.e., without contact with a support) induced by airborne methyl jasmonate (MeJA). (A) Free coiling after mechanical stimulation. (B) Free coiling after incubation in an atmosphere of MeJA (calculated concentration of MeJA in the atmosphere, $\sim 0.7 \mu\text{M}$). (C) Shoot tip specimens were placed in water and incubated for 16 h in two separate chambers: one chamber contained a cotton plug dipped in a methanolic solution of MeJA at a distance of 10 cm from the specimen (left) and the other chamber contained a cotton plug dipped in the solvent alone (right). Note that in the specimen exposed to MeJA vapors, the youngest tendril (triangle) shows only slight bending, whereas the other two tendrils (arrows) have coiled into a tight spiral. Tendrils in the control specimen remained straight. From Falkenstein *et al.* (1991).

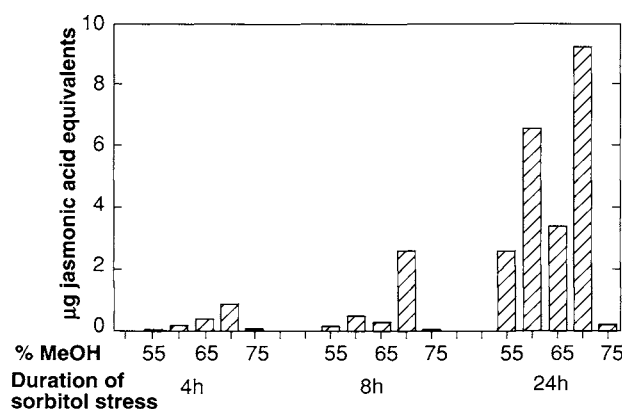


FIGURE 12-5 Effect of water stress on endogenous concentrations of jasmonates. Barley leaf segments were floated on 1.0 M sorbitol for increasing lengths of time. Such treatment with sorbitol caused leaf cells to lose water, causing dehydration stress. The leaves were extracted with MeOH concentrations ranging from 50 to 75% after 4, 8, or 24 h of stress, and endogenous JAs and their metabolites were measured by radioimmunoassay. From Parthier *et al.* (1992) with kind permission from Kluwer.

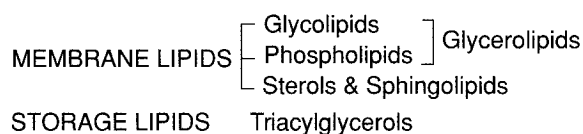
BOX 12-2 FATTY ACIDS AND MEMBRANE AND STORAGE LIPIDS

FATTY ACIDS OCCUR in plants as constituents of membrane lipids and storage lipids; small amounts may also occur free. Membrane lipids in plants and algae are mainly glycerolipids; sterols and sphingolipids provide minor components (Fig. 12-6). Glycerolipids are of two main types: glycolipids, which are the major membrane lipids in chloroplasts (or plastids), and phospholipids, which are the major constituents of membranes in other parts of the cell. Storage lipids, synthesized in organs such as seeds, are exclusively triacylglycerols (TAGs).

Fatty acids differ from each other in the number of C atoms, from 12-C to 24-C, and in their degree of unsaturation, from none, one, two, three, or four. Three unsaturated fatty acids—oleic acid (18:1), linoleic acid (18:2), and linolenic acid (18:3)—predominate in plants. Whereas fatty acids in membranes are restricted to a few types because of steric and structural reasons, storage lipids show a great variety of fatty acids.

A

Major types of Lipids



B

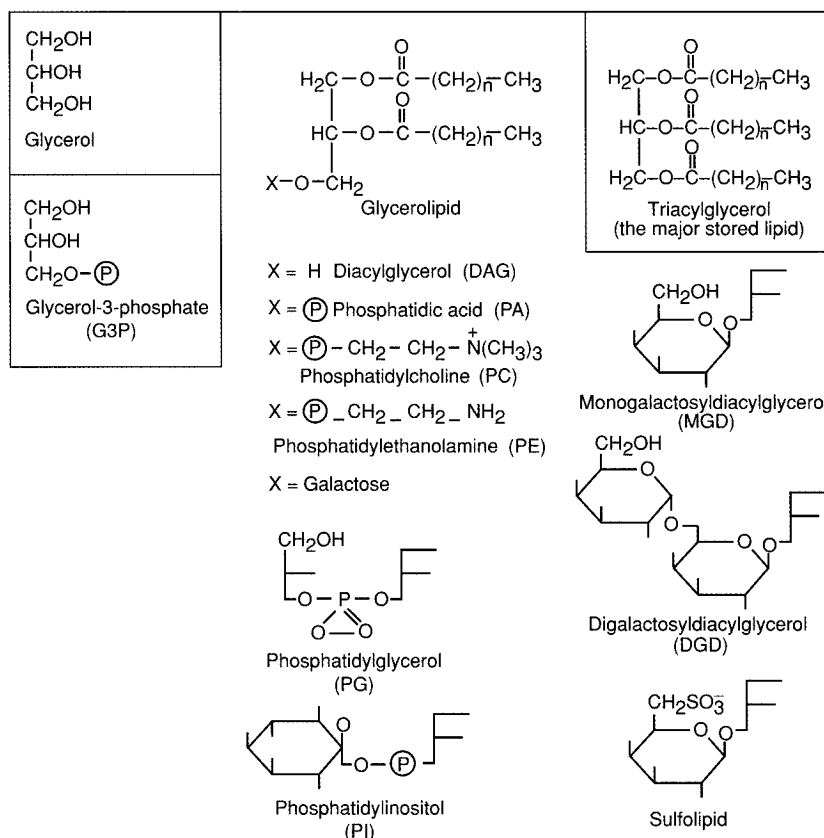


FIGURE 12-6 Major types of plant lipids (A) and structures of glycerol, glycerol-3-phosphate, some glycerolipids, and triacylglycerol (B).

Synthesis of Fatty Acids The synthesis of fatty acids in plants occurs exclusively in the plastids and proceeds in a series of cyclic reactions starting with the conversion of acetyl-CoA to malonyl-CoA. Two carbon atoms from the acetate moiety are condensed with malonyl-ACP and, again, at each successive condensation step in the cycle. The growing fatty acid chain is tethered to an acyl carrier protein (ACP), which provides aqueous solubility. The cycle continues until the chain is 16 or 18C long. Some 16:0-ACP is released from the fatty acid synthesis machinery, but most is elongated to 18:0-ACP and is converted to 18:1-ACP by a specific desaturase. Thus, 16:0 and 18:1 are the major products of fatty acid synthesis in plastids. Enzymes involved in the synthesis, some seven enzymes, catalyze individual steps and occur as a complex, referred to as fatty acid synthase, in the stroma of the plastid. This mode of fatty acid synthesis differs from that in animals and fungi, where the reactions are catalyzed by multifunctional enzyme complexes resident in the cytosol.

Synthesis of Membrane Lipids Fatty acids are esterified to glycerol to give rise to glycerolipids. The major glycerolipids in membrane are synthesized by two parallel pathways. One, referred to as a prokaryotic pathway, operates in the plastids and gives rise to glycolipids; the other, referred to as the eukaryotic pathway, operates in the cytoplasm on ER membranes and gives rise to phospholipids. Some products of the eukaryotic pathway move back into the plastids and contribute lipids to the chloroplast membranes.

The Prokaryotic Pathway The fatty acid components of 16:0-ACP and 18:1-ACP are esterified with glycerol-3-phosphate (G-3-P) to give rise to diacylglycerol phosphate, or phosphatidic acid (PA) (see Fig. 12-7). PA gives rise to phosphatidylglycerol (PG). It is also dephosphorylated by a specific phosphatase to form diacylglycerol (DAG). DAG gives rise to the glycolipids, monogalactosyldiacylglycerol (MGD) and sulfolipid (SL). MGD condenses with another galactose residue to give rise to digalactosyldiacylglycerol (DGD).

The Eukaryotic Pathway A substantial proportion of the 16:0 and 18:1 fatty acids are released from their ACPs by specific thioesterases and are transferred out of the chloroplast into the cytoplasm (Fig. 12-7). On the cytoplasmic side, they are conjugated to CoA before esterification with glycerol-3-phosphate to give rise to PA. This esterification and other steps are catalyzed by enzymes resident in the endoplasmic reticulum (ER). PA is used for the synthesis of PG and phosphatidylinositol (PI), an important component in signaling (see Chapter 25). PA is also dephosphorylated to produce DAG, which plays a central role in the synthesis of phospholipids, such as phosphatidylethanolamine (PE) and phosphatidylcholine (PC), both important constituents of cellular membranes other than those of plastids. Some DAG and PC are transferred back to the plastids and are converted to galacto- and sulfolipids. The relative proportions of chloroplast membranes that arise *via* the eukaryotic pathway in the cytosol vary in different plants. In *Arabidopsis* and spinach they may account for ~ 50%, whereas in pea and barley they may account for more than 95% of lipids synthesized on the ER.

Specific Desaturases Provide Unsaturation to Fatty Acids *in Situ* Desaturase enzymes provide unsaturations at specific locations in the fatty acid chains. For example, a specific desaturase catalyzes the conversion of 18:0 to 18:1 by introducing a double bond at $\Delta 9$ in a *cis* configuration. Another desaturase adds a double bond at $\Delta 12$ of 18:1 to give rise to 18:2, and so on. These unsaturations are thought to be carried out following attachment of the various head groups to the diacylglycerol moiety while the fatty acids are esterified to the glycerol backbone. Different desaturases catalyze similar unsaturations in plastids and cytosol. Most are integral membrane proteins that utilize glycerolipids as substrates. Many mutants defective in fatty acid desaturase enzymes (*fad* mutants) are known from *Arabidopsis* and, along with labeling studies, have helped in elucidation of the lipid biosynthetic pathways. Some of these mutants are listed in Table 12-2.

Linolenic acid (18:3), the precursor for the octadecanoid pathway for the biosynthesis of jasmonates, is thought to be derived from phosphatidylcholine in the ER or galactolipids or phosphatidylglycerol in chloroplast membranes.

Synthesis of Storage Lipids DAG in the cytosol also gives rise to storage lipids, which are exclusively triacylglycerols (TAGs). The stereospecific distribution of fatty acids on a TAG molecule is not random—the unsaturated fatty acids usually occur in the *sn*-2 position, whereas the *sn*-1 and *sn*-3 positions contain both saturated and unsaturated acyl groups. The last enzyme of TAG assembly, and unique to it, diacylglycerol acyltransferase, is found in the microsomal fraction. A mutant specifically defective in diacylglycerol transferase in *Arabidopsis* has been identified. The mutant produces very little triacylglycerols, but sterol synthesis proceeds normally.

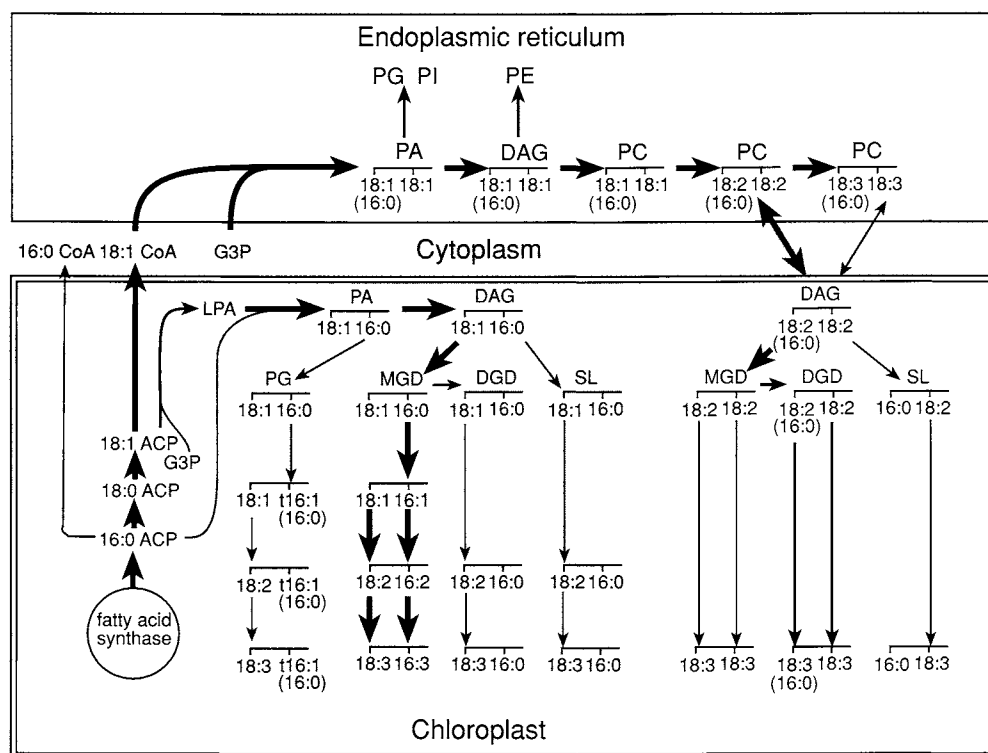


FIGURE 12-7 Summary diagrams illustrating the relationships among synthesis of fatty acids, membrane lipids, and storage lipids. The set of reactions in the shaded area represents the prokaryotic pathway; sets outside the shaded area are part of the eukaryotic pathway. Fatty acids are synthesized in the plastid primarily as 16:0 and 18:1 by the enzyme complex, fatty acid synthase. Lysophosphatidic acid (LPA) is formed by the esterification of 18:1 to the *sn*-1 position in glycerol-3-phosphate (G-3-P). In the prokaryotic pathway, 16:0 is esterified to the *sn*-2 position in LPA to give rise to PA. PA is used for the synthesis of phosphatidylglycerol (PG) or diacylglycerol (DAG). DAG subsequently gives rise to mono- and digalactodiacylglycerols (MGD and DGD) and also sulfolipids (SL). A large proportion of 18:1 and 16:0 fatty acids are exported from the chloroplast as free fatty acids, converted to acyl-CoA esters on entry into the cytoplasm, and then utilized for lipid synthesis by acyltransferases located in the ER. PA and DAG are important intermediates in the eukaryotic pathway as well. PA is used for the synthesis of PG, as well as phosphatidylinositol (PI), an important component in signaling (see Chapter 25). DAG is the branch point for the synthesis of membrane phospholipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE). In green leaves, depending on the species, 50–95% of the lipid synthesized in the ER is transported ultimately back to the chloroplast. In seeds and other lipid-storing tissues, DAG is used predominantly for the synthesis of storage lipids or triacylglycerols (TAGs). Lipids synthesized within the ER always have an 18 carbon fatty acid at the *sn*-2 position of the glycerol moiety, whereas lipids synthesized in the chloroplast always have a 16:0 at the *sn*-2 position. Adapted with permission from Somerville and Browse (1996), © Elsevier Science.

TABLE 12-2 Fatty Acid Desaturases from *Arabidopsis*^a

Mutant gene	Substrate	Location	Precursor	Product
<i>fab2</i>	acyl-ACP	Chloroplast	18:0	18:1(9c)
<i>fad2</i>		ER	18:1(9c)	18:2(9c,12c)
<i>fad3</i>		ER	18:2(9c,12c)	18:3(9c,12c,15c)
<i>fad4</i>	PG	Chloroplast	16:0	16:1(3t)
<i>fad5</i>	GL, SL	Chloroplast	16:0	16:1(7c)
<i>fad6</i>	GL, SL, PG	Chloroplast	16:1(7c)	16:2(7c,10c)
			18:1(9c)	18:2(9c,12c)
<i>fad7</i>	GL, SL, PG	Chloroplast	16:2(7c,10c)	16:3(7c,10c,13c)
			18:2(9c,12c)	18:3(9c,12c,15c)
<i>fad8</i>	GL, SL, PG	Chloroplast	16:2(7c,10c)	16:3(7c,10c,13c)
			18:2(9c,12c)	18:3(9c,12c,15c)

^a ACP, acyl carrier protein; c, *cis*-olefinic; ER, endoplasmic reticulum; GL, galactolipid; PG, phosphatidylglycerol; SL, sulfolipid; t, *trans*-olefinic. Reprinted with permission from Somerville and Browse (1996), © Elsevier Science.

4.1. Main Steps

Jasmonic acid is derived from linolenic acid (LA), a C₁₈ fatty acid with three unsaturations (18:3), which may occur free or be released from membranes or storage lipids by the action of lipases. LA is oxidized by a lipoxygenase to form 13-hydroperoxylinolenic acid (13-HPLA), which is a branch point for several pathways leading to the synthesis of defense-related compounds. In one pathway, catalyzed by an allene oxide synthase (AOS) and an allene oxide cyclase (AOC), 13-HPLA gives rise to an intermediate, 12-oxo-phytodienoic acid (12-oxo-PDA). The 12-oxo-PDA undergoes reduction and three successive β oxidations, which shorten the side chain by two carbons at each step, to give rise to JA. A simplified scheme is shown in Fig. 12-8. The (+)-7-iso-JA is formed first, but, as stated earlier, epimerizes to the more stable *trans* configuration of the (–)-JA. In another pathway, 13-HPLA is cleaved by a hydroperoxy lyase (HPL) to yield a C₆ and a C₁₂ compound. These compounds give rise to volatile aldehydes and 12-oxo-acids, which act in defense against insects and pathogens and in wound healing. A third pathway catalyzed by peroxygenase leads to the synthesis of cutin monomers (which polymerize on epidermal cell surface to yield cuticle). Since many compounds in the pathway just described are C₁₈ compounds, the pathway is referred to as the octadecanoid pathway. The compounds synthesized are referred to as oxylipins. Oxylipins are a family of defense-related compounds derived from the breakdown of polyunsaturated fatty acids in plants and animals. In plants, some oxylipins, such as jasmonic acid, also act as growth regulators.

4.2. Enzymes and Their Genes

Lipoxygenases are nonheme, iron-containing dioxygenases that catalyze the addition of molecular oxygen to free polyunsaturated fatty acids. This oxidation occurs in a highly stereospecific manner at C-9 or C-13 to yield 9- or 13-hydroperoxy derivatives of fatty acids (Fig. 12-9). In plants, linoleic acid (18:2) and linolenic acid (18:3) are the natural substrates [in animal systems, arachidonic acid (18:4) is the main substrate]. The C-9 hydroperoxy derivatives are involved chiefly in the synthesis of volatile aldehydes and oxoacids. The 13-hydroperoxy derivatives may give rise to volatile derivatives or, alternatively, form JA after AOS and AOC catalyzed cyclization, followed by reduction and β oxidation. Different LOX isoforms catalyze C-9 or C-13 oxidation.

LOXs are encoded by multigene families. Several different LOX isoforms occur in a single plant, sometimes belonging to two or more families, and are accumulated in a tissue/organ-specific manner by developmental and/or environmental cues. In soybean, at least seven LOXs are known. These isoforms differ in their pH optima, some are active at acidic pH, whereas others are active at neutral pH; in their requirement for calcium for activation; in their substrate specificity; and in specific reaction products. Some are seed specific, whereas others are more specific to vegetative parts. In addition, many lipoxygenases are accumulated as vegetative storage proteins (see Chapter 20), although why an enzyme should be stored in this manner is not clear.

LOX protein and activity levels are enhanced in plant tissues in response to a variety of stimuli, wounding,

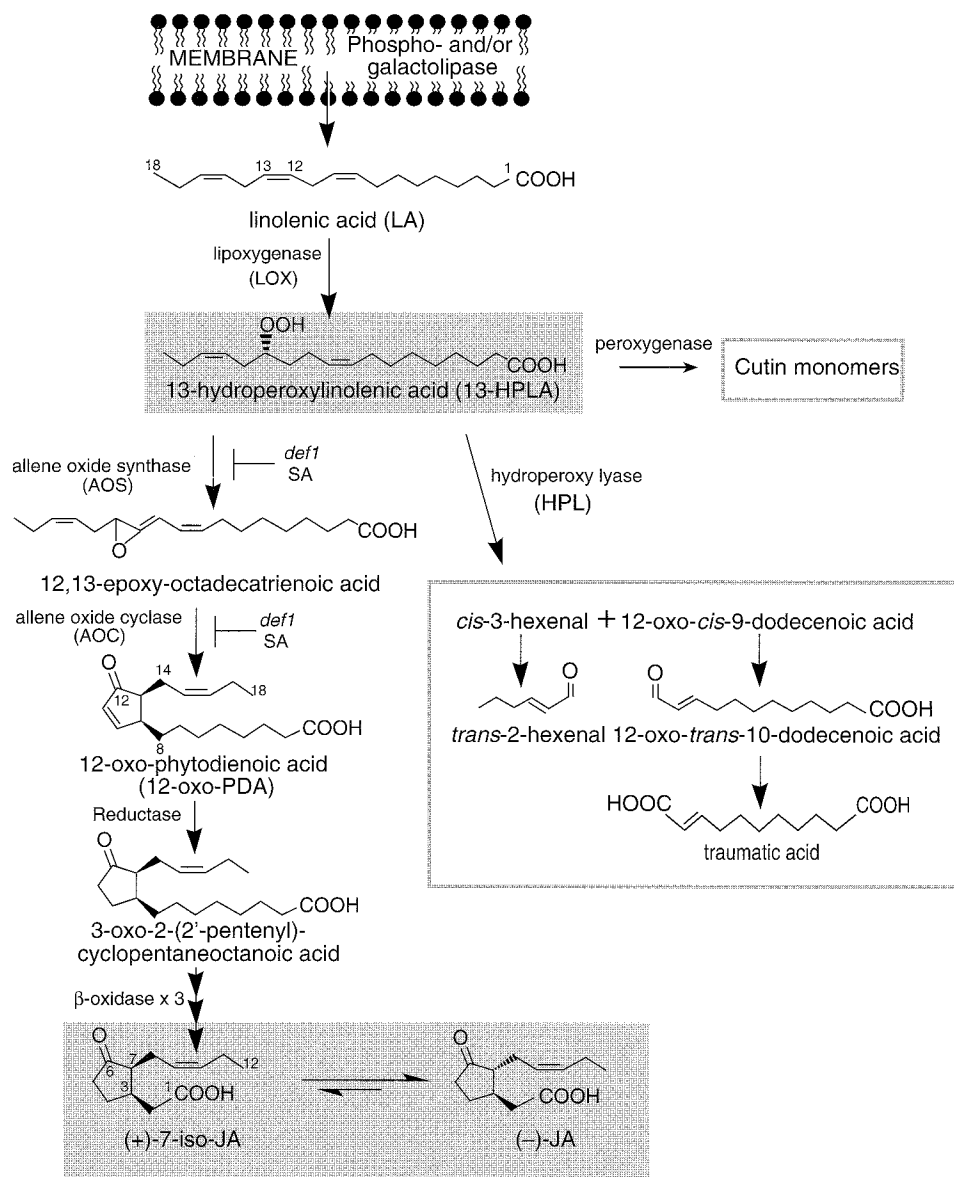


FIGURE 12-8 The octadecanoid pathway of jasmonic acid biosynthesis. Linolenic acid is released from membrane phospholipids or galactolipids by phospho- or galactolipase action. Storage lipids may also serve as a source. It is oxidized by a lipoxygenase (LOX) to 13-hydroperoxylinolenic acid. The latter compound, after several steps, including cyclization, reduction, and β oxidations, gives rise to (+)-7-iso-JA, which isomerizes to (-)-JA. 13-Hydroperoxylinolenic acid, by alternate pathways, gives rise to C_6 aldehydes and C_{12} oxo-acids or to cutin monomers. A tomato mutant, *def1*, is blocked in one of the steps in the production of 12-oxo-PDA from 13-HPLA. Salicylic acid (SA) is also thought to inhibit one of those steps. Adapted from Blée and Joyard (1996).

pathogen attack, treatment of cultured cells with elicitors, water stress, treatment with ABA and IAA, and, most notably, treatment with JA or MeJA.

LOX cDNAs and genes from several plants (e.g., soybean, *Arabidopsis*, potato, barley) have been cloned and are providing a clearer insight into the diversity of LOX enzymes and their specific functions. Sequence comparisons of deduced amino acids indicate that

while the isoforms vary in their N termini, they share high sequence similarity in other regions of the protein, including the active site with iron and the carboxy terminus. Variability in the N terminus explains, in part, the diversity of inducing stimuli and the tissue/organ specificity of expression.

13-HPLA in two closely linked steps gives rise to 12-oxo-PDA (see Fig. 12-8). The first enzyme, an allene

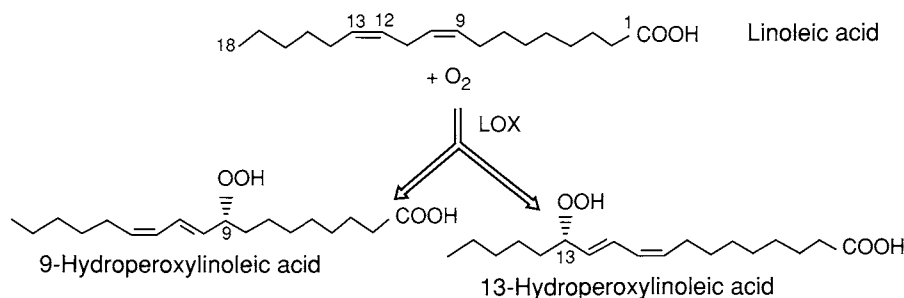


FIGURE 12-9 Oxidation of linoleic acid (18:2) by lipoxygenase giving rise to either 9- or 13-hydroperoxylinoleic acid. Adapted from Siedow (1991).

oxide synthase (AOS), is a cytochrome P450-type monooxygenase, and its gene (AOS) has been cloned from several plants (e.g., flax, guayule, *Arabidopsis*). AOS cDNA from flax, expressed in potato plants under the control of a constitutive promoter (CaMV S35), raised endogenous JA levels 6- to 12-fold higher than in untransformed plants and, when wounded or put under water stress, showed still higher levels of JA (Fig. 12-10). Experiments using *Arabidopsis* reveal another interesting feature. Treatment of tissues with MeJA, or the methyl ester of 12-oxo-PDA (Me-PDA), enhances the levels of both the AOS protein and its mRNA. Thus, jasmonates not only upregulate some genes encoding lipoxygenases, they also upregulate AOS genes. A gene encoding the reductase enzyme, which converts oxo-PDA to 3-oxo-2-(2'-pentenyl)-cyclopentanoctanoic acid in *Arabidopsis*, has also been cloned.

As mentioned earlier, 13-HPLA is a branch point for the synthesis of JA or C₆ aldehydes and C₁₂ oxoacids. The latter compounds are produced via the hydroperoxy lyase (HPL) pathway. The HPL cDNA has been cloned and its expression is also upregulated by exposure to MeJA.

In summary, jasmonates bring about their own synthesis, as well as that of aldehyde volatiles, in a two-pronged strategy for defense. They do so by inducing some key enzymes (LOX, AOS, HPL) in their own synthesis and that of volatiles. Different LOX isoforms are involved in the determination whether the allene oxide pathway for JA or the HPL pathway for aldehydes and oxoacids is favored. Although not reviewed here, it is also likely that different LOX isoforms are involved in the production of JA after water stress and after wounding or exposure to microbial organisms.

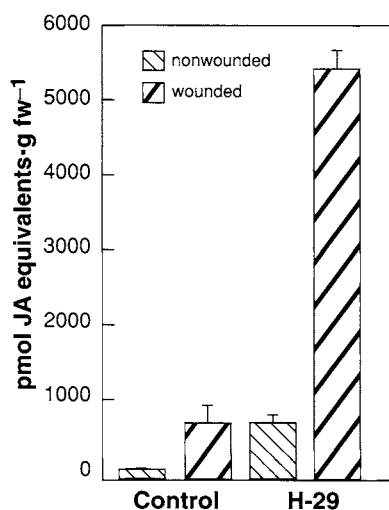


FIGURE 12-10 Concentrations of JA in a transgenic potato transformed with the coding sequence of an allene oxide synthase cDNA from flax. Levels in unwounded and wounded transformed plants and in untransformed control are indicated. Results from only one set of transformations (H-29) are shown. JA was isolated from leaves and quantified using polyclonal Abs and ELISA. Modified from Harms *et al.* (1995).

4.3. Sites of JA Biosynthesis

In higher plants, the steps in the conversion of LA to 12-oxo-PDA to JA have been shown to occur in leaf tissue, young immature fruits, cotyledons of germinated seeds, and coleoptiles. Thus, enzymes for JA synthesis are present in nearly all parts of the plant. As to intracellular location, there are many reports of LOX enzymes being localized in chloroplasts. Enzymes associated with the metabolism of 13-HPLA, such as AOS and HPL, are associated with chloroplast membranes. Moreover, the deduced amino acid sequence of an AOS cDNA indicates the presence of a signal peptide targeting it to plastids, and immunoblots against the AOS protein indicate that it is localized in plastids. In a careful study using membrane and stromal fractions from chloroplasts of spinach, it was shown that all the major enzymes in the synthesis of oxylipins, LOX, HPL, and AOS, are localized in membrane fractions from the chloroplast envelope, not stroma fractions. Thus, the early steps in JA biosynthesis occur in plastids. The location of AOC and other enzymes that cata-

lyze subsequent steps is unclear, but β -oxidation is believed to occur in peroxisomes.

5. JA SYNTHESIS MUTANTS

JA synthesis mutants are known from tomato, *def1* (for defenseless), and *Arabidopsis*, *fad3-2 fad7-2 fad8* (fatty acid desaturase deficient). The site of *def1* mutation is indicated in Fig. 12-8. It appears to be blocked in the conversion of 13-HPLA to 12-oxo-PDA, because the application of 12-oxo-PDA or JA restores the mutant to the wild type, but the application of 13-HPLA does not. The DEF1 protein could be an allene oxide synthase or allene oxide cyclase. The *fad3-2 fad7-2 fad8* mutant in *Arabidopsis* is inhibited in the production of linolenic acid from the precursor linoleic acid in membrane lipids (see Table 12-2). Both of these mutants show very low levels of endogenous JA, which do not increase substantially after wounding (Fig. 12-11). They are literally defenseless against their insect pests, but exogenous application of JA or exposure to MeJA vapor restores their defense.

6. JA SYNTHESIS INHIBITORS

Salicylic acid (SA), its acetylated derivative, aspirin (ASA), and diethyldithiocarbamic acid (DIECA) are potent inhibitors of JA biosynthesis and, as expected, inhibit the expression of JA-induced genes (Fig. 12-12). To see which step is blocked, the methodology is to apply the inhibitor together with one or another precursor in the JA biosynthetic pathway and monitor the expression of a JA-induced gene, such as proteinase inhibitor I or II [*Pin1* or *Pin2* (*PIN1* or *PIN2*)]. Application of aspirin together with linolenic acid or 13-HPLA was not able to restore *Pin2* expression, but that of 12-oxo-PDA or JA was able to. This shows that aspirin was blocking a step between 13-HPLA and 12-oxo-PDA (see Fig. 12-8). Aspirin and other related benzoic compounds are known to inhibit the cyclooxygenase activity of the enzyme prostaglandin endoperoxide synthase in animal systems.¹ Because the conversion of 13-HPLA to 12-oxo-PDA involves a cyclase (AOC),

¹Jasmonic acid is structurally similar to eicosanoids (prostaglandins and leukotrienes), which are released during inflammation and injury reactions in animal systems. Similar to JA, prostaglandins and leukotrienes are derived from polyunsaturated fatty acids, and the first step in the synthesis of leukotrienes is catalyzed by a LOX and of prostaglandins by a cyclooxygenase. Aspirin and other related benzoic compounds inhibit the cyclooxygenase activity of the enzyme prostaglandin endoperoxide synthase. Thus, they act as potent non-steroidal anti-inflammatory drugs (NSAIDs).

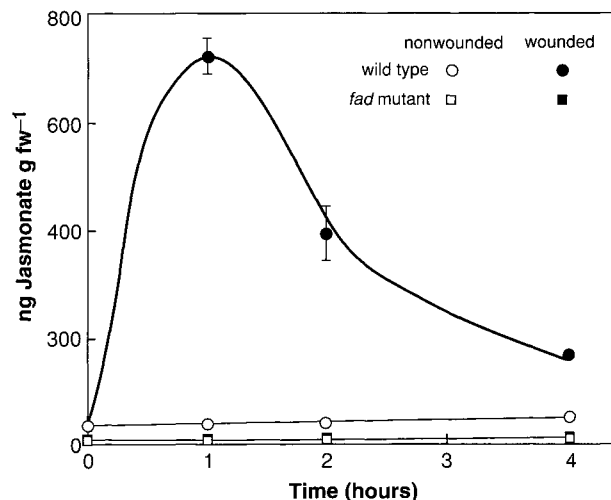


FIGURE 12-11 Kinetics of jasmonate accumulation in leaves of *Arabidopsis* wild-type and *fad3-2 fad7-2 fad8* mutant after wounding. Reprinted with permission from McConn *et al.* (1997), © National Academy of Sciences, USA.

aspirin, most likely, inhibits the latter cyclization step. DIECA also inhibits the step(s) between 13-HPLA and 12-oxo-PDA. Its mode of action is not known, but it is a strong reducing agent and may divert 13-HPLA to some other derivative, thus making it unavailable for cyclization to 12-oxo-PDA.

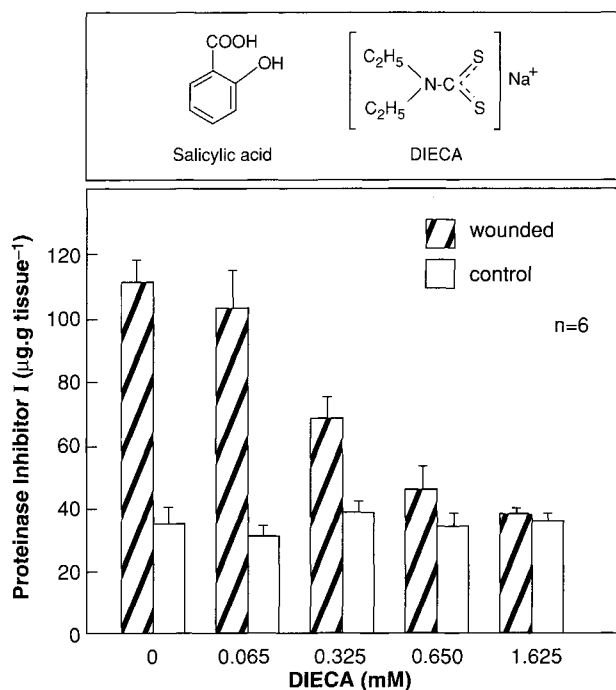


FIGURE 12-12 Structures of salicylic acid and diethyldithiocarbamic acid (DIECA), and inhibition of accumulation of proteinase inhibitor I, a defense-related protein, in tomato leaves after application of DIECA. From Farmer *et al.* (1994).

7. REGULATION OF ENDOGENOUS LEVELS OF JA

7.1. Endogenous Levels of JA Vary Considerably

Jasmonates are present in all organs of a plant, but the endogenous levels of JA vary considerably (from 10 ng to as much as $3 \mu\text{g} \cdot \text{g fw}^{-1}$), depending on the developmental state of the plant or tissue and the environment. They are usually high in young growing leaves and stems, in reproductive axes, in immature fruit pods, and in seeds than they are in the mature parts of stems and roots. For reasons that are not clear, the growth of young tissues is not inhibited, despite relatively high concentrations of endogenous JAs in these tissues. As indicated earlier, JA levels increase severalfold in response to wounding (see Table 11-1) or attack by pests or pathogens. They also rise under conditions of osmotic stress or water deficit (see Fig. 12-5). However, high levels of endogenous JA by themselves are not enough to promote defense responses in plants. In potato plants overexpressing AOS cDNA, JA levels rose to 600–1400 pmol $\cdot \text{g fw}^{-1}$, about 8–12 times as high as in the untransformed controls, but wound-induced or water stress-induced genes were not expressed unless the plants were wounded or water stressed. In barley leaves, a rise of endogenous jasmonic acid resulting from sorbitol stress did not induce the mRNA for a 100-kDa LOX involved mostly in the production of volatile aldehydes and oxoacids.

7.2. Regulation of JA Biosynthesis

Kinetic data indicate that an increase in JA production occurs almost immediately after wounding (see curve for wild-type plants in Fig. 12-11), whereas the increase in LOX enzyme activity or mRNA occurs considerably later. This observation has led to the suggestion that some LOX isoforms may be expressed constitutively.

Exogenous application of linolenic acid (LA) to plant tissues results in accumulation of JA. Wounding of plant tissues or treatment of cell cultures with fungal elicitors causes an increase in phospholipase activity and an increased supply of LA, which, in turn, leads to an increase in JA content, although the correspondence may not be one to one (Fig. 12-13).

JA biosynthesis may also be regulated at the level of 13-HPLA. As mentioned earlier, there are multiple isoforms of LOX enzymes, which may be differentially induced after different treatments. Such differential induction may determine whether 13-HPLA will give rise to JA *via* the action of allene oxide synthase and cyclase

enzymes or be diverted toward volatile aldehyde synthesis *via* the activity of hydroperoxy lyase. Still another site for regulation may be at the epimerization step. The molar ratios of (–)-JA and (+)-iso-JA *in vitro* are ~9:1. In *Vicia faba* fruit pods, they were reported to be 2:1. Thus, it is possible that different tissues may regulate the levels of the active isomer by regulating the rate of epimerization. Finally, many metabolites and derivatives of JA are known from plant tissues, and these may further help regulate the endogenous levels of JA.

7.3. JA-Related Compounds and JA Metabolites

The number of JA-related compounds and their metabolites in plants, referred to as jasmonates, is large because many isomers are possible (Fig. 12-14). Not all of these products are formed in any one plant. Some of these compounds and their isomers are probably formed along with JA during the biosynthetic pathway, whereas others are produced as metabolites of JA.

Many products derive from modifications at C-1, C-6, C-11, or C-12. The major metabolite of JA, methyl jasmonate (see compound V in Fig. 12-1), is formed by methylation of the C-1 carboxyl group. Other modifications at C-1 include glucosylation with sugar residues or conjugations with amino acids (compound I in Fig. 12-14).

C-11- or C-12-hydroxylated JAs and their glucosyl conjugates (e.g., II, III) occur in many plants. The 12-OH-(+)-7-iso-JA, known as **tuberonic acid**, was reported some years ago to promote tuber formation in cuttings of potato (see Chapter 20). **Cucurbic acid** (IV), where the C-6 keto group is reduced, was isolated from cucumber seeds (hence, the name). It is reported to be a potent inhibitor of growth in the rice seedling bioassay and, also along with JA, is believed to induce tuberization in potato. Cucurbic acid may also be glucosylated (V).

How these compounds are formed and the enzymes involved are unknown. The functions of these compounds, except MeJA and possibly tuberonic acid and cucurbic acid, are also unknown. Structure/activity relationships for jasmonic acid isomers and substituted products indicate that the C-1 acetyl group, the R absolute configuration at C-3, the pentenyl side chain inserted at C-7, and the keto or hydroxyl group at C-6 are necessary for the biological activity of JA or MeJA. Hydroxylated or glucosylated derivatives, as well as amino acid conjugates, are formed when endogenous levels of JAs reach a high level. This can be shown by supplying radiolabeled JA to plant tissues or cell suspension cultures followed by

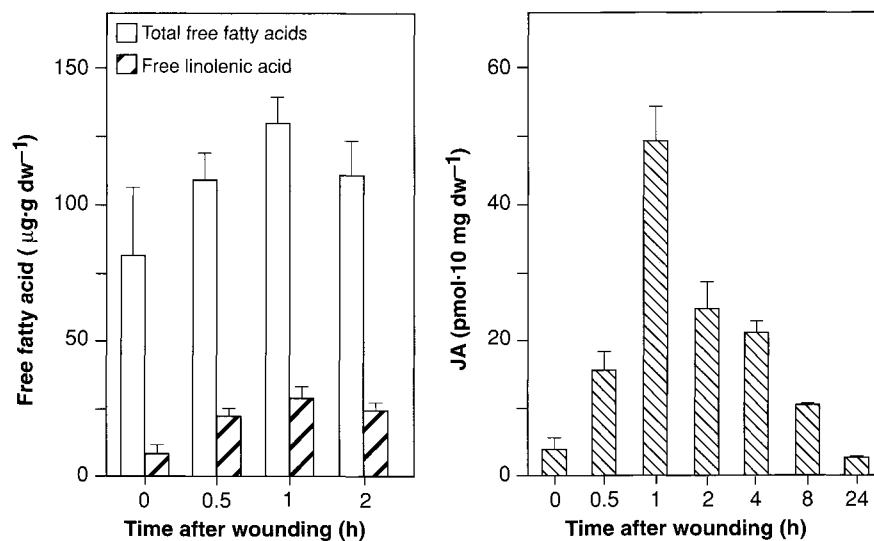


FIGURE 12-13 Rise in levels of total free fatty acids, free linolenic acid, and JA in tomato leaves after wounding. From Conconi *et al.* (1996).

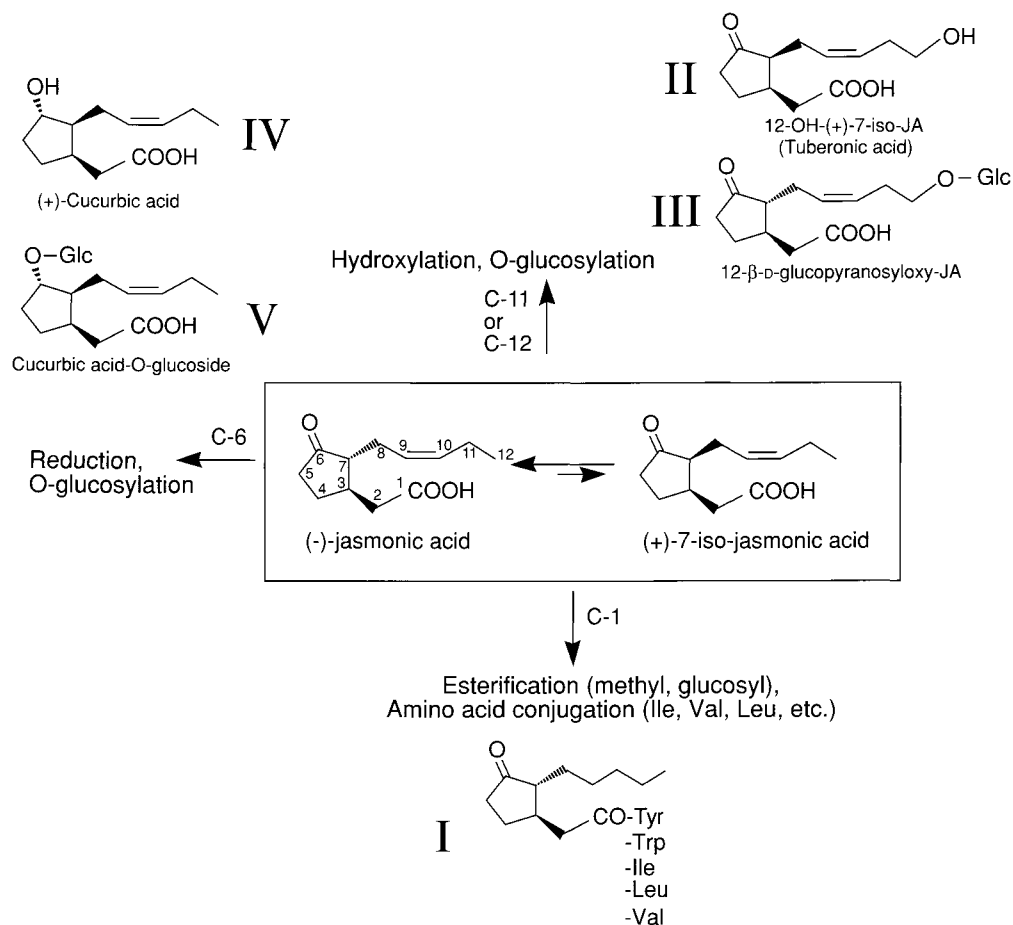


FIGURE 12-14 Structures of some JA-related compounds and JA metabolites. From Parthier *et al.* (1992).

analysis of labeled products. It can also be shown by putting leaf segments under water stress and analyzing the accumulated JA and its metabolites (see Fig. 12-5). Thus, they may play a role in regulating the endogenous levels of JA. Methyl jasmonate occurs in small quantities and, being volatile, probably escapes into the atmosphere. Many of the metabolites are more polar than JA and could be used for storage in vacuoles and/or transport of JA. However, there is little evidence that such storage or transport occurs. Also, it is unclear whether JA can be reversibly obtained from these derivatives.

The structure and metabolism of JA would not be complete without a mention of traumatin. Wounding causes synthesis of defense-related proteins and it also induces cell division and wound repair machinery. Reports of a "wound" hormone, which induces cell divisions at wound sites, go back to the 19th century, and a specific bioassay, known as Wehnelt bean test, was devised to test the effects of potential candidates for the wound hormone. In 1939, a compound was isolated from the mesocarp of wounded bean plants and was shown to enhance cell proliferation at the wound site; it was given the trivial name **traumatin**. Pure crystals of traumatin were obtained and identified as *trans*-2-dodecenedioic acid, or **traumatic acid**. Traumatin has been reported to induce wound periderm formation in potato tuber discs, to promote tumor formation in green tomatoes, and to accelerate cell proliferation in the abscission zone in petiolar explants from cotyledons of cotton. However, many other tissues fail to respond. In 1979, a careful study by Zimmerman and Coudron showed that one of the products formed after the cleavage of 13-hydroperoxylinoleic acid by the enzyme hydroperoxy lyase, 12-oxo-*trans*-10-dodecenoic acid (*trans*-10-ODA, see Fig. 12-8), has the same activity as traumatin in the Wehnelt bean bioassay. However, despite the possibility that it could give rise to traumatic acid by dehydrogenation, it was not a precursor of traumatin. The origin of traumatin is still unclear. Meanwhile, not only *trans*-10-ODA, but many other fatty acids and their esters, including the C₁₂ saturated monocarboxylic lauric acid, have traumatin-like activity in the Wehnelt bean assay.

nase inhibitors and chitinases, and volatile aldehydes and oxoacids. Jasmonates share many activities with ABA, including inhibition of growth of stems and roots, inhibition of seed germination, and induction of some seed storage protein genes. They also share some responses with ethylene in fruit ripening and in senescence and abscission. Jasmonic acid is synthesized from an unsaturated fatty acid, linolenic acid, via 13-hydroperoxylinolenic acid and 12-oxo-PDA. The production of 13-HPLA involves the activity of lipoxygenases, which are a superfamily of enzymes with many isoforms and a diversity of functions. Some isoforms may be expressed constitutively, whereas others are induced by a variety of stimuli, including wounding, water stress, and treatment with hormones such as ABA, IAA, or JA/MeJA. Some LOX isoforms seem to be more specific for the production of JA; others for the production of aldehydes and oxoacids. The key enzymes in these two pathways, allene oxide synthase and hydroperoxy lyase, are also induced by JA/MeJA. Mutants in JA biosynthesis are known from tomato and *Arabidopsis*; both mutants are defenseless against insect herbivores because they cannot synthesize defense-related proteins. Salicylic acid, acetyl salicylic acid, and DIECA are potent inhibitors of JA biosynthesis and probably inhibit the AOS/AOC-catalyzed conversion of 13-HPLA to 12-oxo-PDA. Jasmonic acid is synthesized in all parts of the plant, and although the intracellular sites are not known fully, several synthesis enzymes, e.g., LOX, AOS, and HPL, seem to be localized in the chloroplasts. Many related compounds or JA metabolites occur naturally in plants. Except for some, their roles are uncertain. Tuberonic acid may have a role in tuber formation in crops such as potato and yam. Cucurbitic acid, like JA, inhibits cell growth and is also reported to induce tuberization. A JA-related compound, 12-oxo-*trans*-10-dodecenoic acid, derived via the HPL pathway, acts similarly to traumatic acid in promoting cell divisions in a bioassay, but many other fatty acids and their derivatives also give a positive response in the bioassay. The role of these metabolites and related compounds in the regulation of endogenous levels of jasmonic acid is unclear.

8. CHAPTER SUMMARY

Jasmonic acid and its methylated derivative, methyl jasmonate, play major roles in plant defense against insect herbivores and microbial pathogens. They do so by inducing the synthesis of enzymes such as protei-

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1. MICROBIAL ASSOCIATIONS WITH PLANTS

A number of microbes, cyanobacteria, eubacteria, and fungi have evolved together with plants and live in association with them. In some cases, there may be no evident symbiotic relationship, but the microbes stimulate growth of the plant, presumably by hormo-

nal secretion, and benefit from improved plant growth. In other cases, there is a specific symbiotic or pathogenic association. N₂-fixing bacteria, such as *Rhizobium* or *Frankia* sp., form symbiotic associations with roots of legumes or other higher plants. The plant roots respond by forming a special organized structure, the nodule, which provides nutrients and shelter to the bacteria, whereas bacteria "fix" or reduce atmospheric N₂ to ammonia, which serves as the nitrogen source for the plant. In still other cases, the association is pathogenic.

At least four classes of phytopathogenic bacteria—*Agrobacterium tumefaciens* and *A. rhizogenes*, *Pseudomonas savastanoi*, *Erwinia herbicola*, and *Rhodococcus fascians* (formerly *Corynebacterium fascians*)—produce neoplastic or hyperplastic diseases in plants (see Tables A2-1 and A2-3). The resulting galls (or tumors) display either completely unorganized growth or growth with abnormal shoot or root development. Among these bacteria, the two species of *Agrobacterium* differ from the rest in that they cause a genetic transformation of the infected host cells, whereas the others do not.

The tumor-producing infections by *Agrobacterium* and *Pseudomonas* have been particularly well studied for several reasons: (i) They synthesize IAA and CK and thus provide a parallel system for studying the biosynthesis of these hormones. (ii) Microbial genes encoding the enzymes involved in these syntheses have been cloned; they can be expressed in higher plants, and the metabolism of the hormones, as well as their specific morphogenetic effects, can be studied. (iii) *Agrobacterium* uses a highly efficient and sophisticated machinery to insert part of its DNA into the host

TABLE A2-1 Microbial Genes Encoding IAA and CK Biosynthesis Enzymes^a

Organism	Hosts (disease)	Oncogenes [location] (hormones)
<i>Agrobacterium tumefaciens</i>	Many dicots and gymnosperms (crown gall)	<i>tms1</i> , <i>tms2</i> , <i>tmr</i> (<i>ipt</i>) [all on T-DNA] <i>tzs</i> [on the Ti plasmid] (IAA, IP, Z)
<i>A. rhizogenes</i>	Many dicots (hairy root)	<i>aux1</i> , <i>aux2</i> , <i>rolA-D</i> [all on T-DNA] <i>tzs</i> [on Ri-plasmid] (IAA, Z)
<i>Pseudomonas syringae</i> pv <i>savastanoi</i>	Oleander, olive (olive knot/gall)	<i>iaaM</i> , <i>iaaH</i> , <i>ipt</i> , <i>ptz</i> [on plasmid or on chromosome] (IAA, Z, diHZ, IP)

^aModified from Morris (1995).

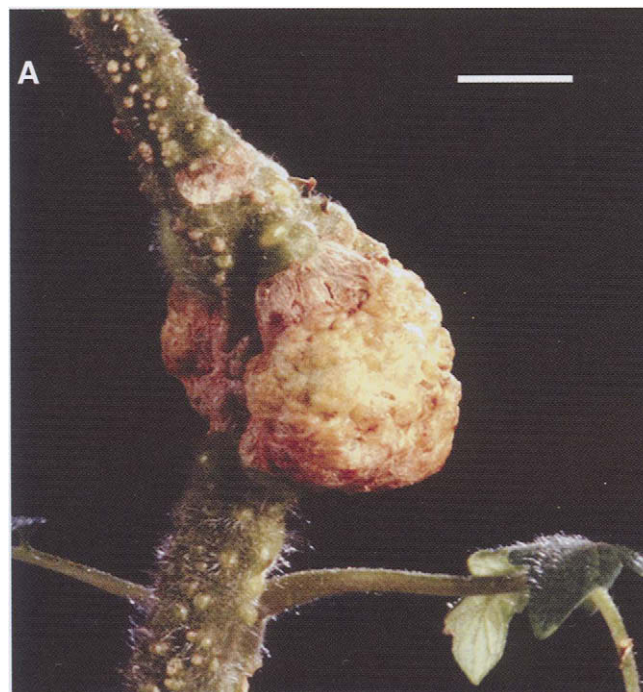
plant. Such transformation is stable in that it is passed on from cell to cell and from generation to generation. We can use the same machinery to insert genes of interest in plants. Indeed, plant transformation using *Agrobacterium* is a standard tool of plant biotechnology. This chapter deals with the synthesis of IAA and CK by these organisms and plant transformation using *Agrobacterium*. Other bacteria and fungi that produce plant hormones are referred to briefly.

2. INFECTION BY AGROBACTERIUM

Agrobacterium tumefaciens and *A. rhizogenes* are free-living soil bacteria in the family Rhizobiaceae, which under certain circumstances become pathogenic. Strains of *A. tumefaciens* form tumors or galls in a large number of dicotyledonous plants and several gymnosperms, and because the tumor is formed at the soil-air transition, the "crown" of the plant, they cause what is known as the crown gall disease (Fig. A2-1). In contrast, *A. rhizogenes* induces excessive root branching, a phenotype known as "hairy." Roots are formed on stems and leaves as well.

2.1. *Agrobacterium tumefaciens* Causes Host Transformation

It had been known since the 1960s that infection by *A. tumefaciens* caused enhanced levels of IAA and cytokinins in the tumor tissue. Furthermore, bacteria-free explants from these tumors, when placed in cul-

**FIGURE A2-1 (A)**

ture, were autonomous in terms of hormonal requirement, i.e., unlike normal tissue, they did not require an exogenous supply of auxins or CKs for proliferation and growth. It was clear that the tissues were producing their own IAA and CKs in a manner that was beyond the control of the host plant. How this came about, however, was not clear until the application of molecular techniques in the 1980s. The mystery was clarified with the identification that the virulent strains of *A. tumefaciens*, in addition to the bacterial chromosome, carry one to several copies of a plasmid, the tumor-inducing or Ti plasmid (Fig. A2-2). On infection, one segment of the Ti plasmid, transfer DNA (T-DNA), gets transferred to the host cell, where it gets incorporated in a stable manner into the host genome. T-DNA carries many genes, including genes that encode enzymes for the synthesis of IAA and cytokinin and for the synthesis of special nutrients for *Agrobacteria*, known as opines.

Opines are condensation products of keto acids or sugars and basic amino acids. Strains of *Agrobacteria* are classified according to the nature of the opine produced. Some seven different groups of opines are recognized. The two main groups are octopine (a condensation product of pyruvate and arginine) and nopaline (a condensation product between arginine and α -ketoglutarate) (Fig. A2-3). Thus, there are octopine-producing strains (e.g., pTiA6) and nopaline-producing strains (e.g., pTiC58). Each strain may produce more than one opine. Thus, octopine-producing

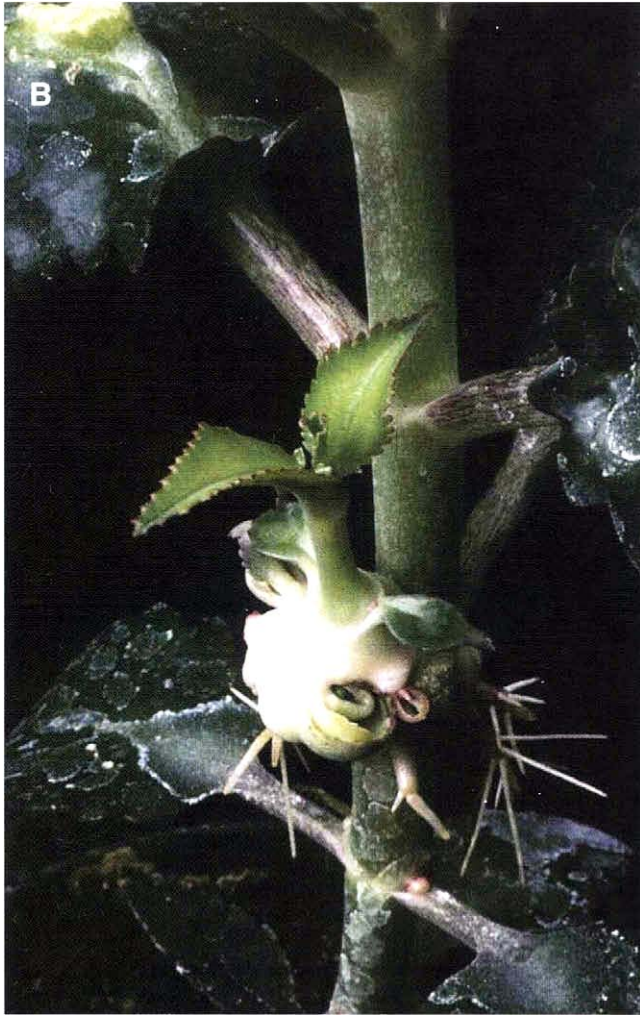


FIGURE A2-1 Crown gall disease of plants. (A) *Agrobacterium tumefaciens*-induced crown gall on a tomato (*Lycopersicon esculentum*) plant. The 2-month old tumor appears in a side view with numerous adventitious roots (white spots on the stem) above and below the crown gall. Leaves show epinasty. Bar segments: 10 mm. Courtesy of Roni Aloni, Aloni *et al.* (1998). (B) Crown gall tumor formed on the stem of a *Kalanchoe* plant after wound infection with *A. tumefaciens* strain C58. Courtesy of Csaba Koncz.

strain pTiA6 may also produce mannopine and agropine.

Although the genes in T-DNA are bacterial genes, they carry all the signals for expression in eukaryotic cells. Thus, their 5' and 3' flanking regions contain "TATA" and "CAAT" boxes and polyadenylation signals, and their transcription is initiated by RNA polymerase II. Thus, they are expressed in plants like plant genes. Once T-DNA gets inserted into the host genome, *Agrobacteria* are no longer necessary for maintaining the infection. This is a case of true transformation, the only known example of transformation in nature involving two kingdoms.

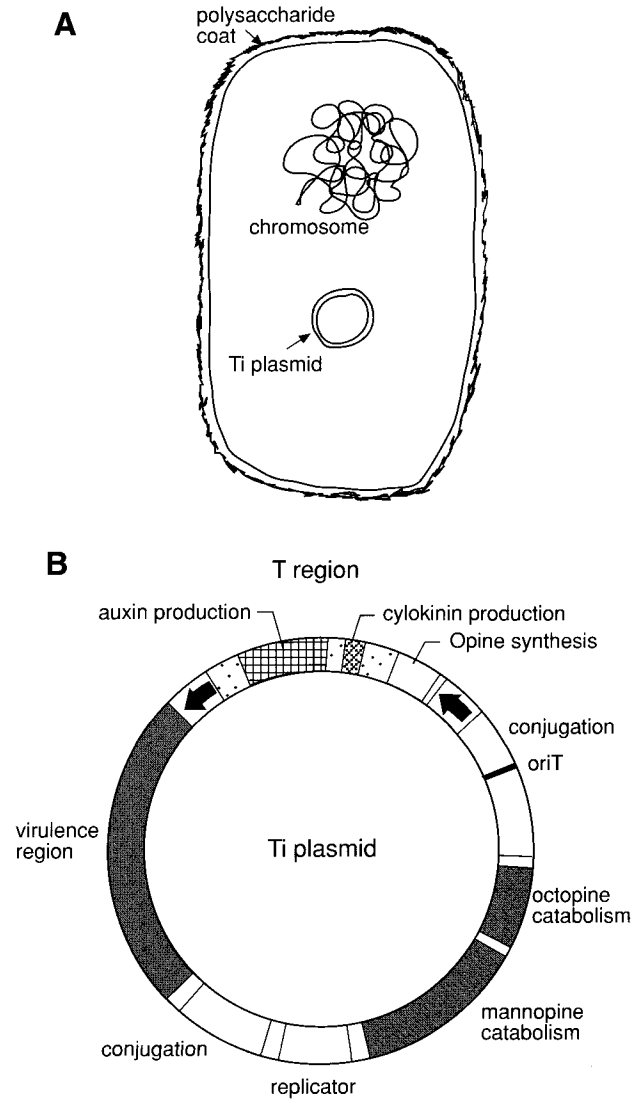


FIGURE A2-2 *Agrobacterium tumefaciens* and the genetic map of the octopine Ti plasmid. (A) A diagrammatic representation of an *Agrobacterium* showing the bacterial chromosome and one copy of a Ti plasmid. The bacterium is surrounded by an extracellular coat of polysaccharides, which plays an important role in establishing contact with the host cell. (B) The octopine Ti plasmid is ~200 kb in size. It is roughly divided into two halves; one half is occupied by replication and conjugation functions, and production of enzymes needed for octopine catabolism. This half has nothing to do with infectivity or genetic transformation. The other half carries T-DNA (T-region) and the *vir* (virulence) region. T-DNA is delineated by a right (R) and a left (L) border (heavy block arrows), and carries the genes for IAA and CK biosynthesis, as well as genes encoding enzymes for opine biosynthesis. The *vir* region carries genes that encode proteins involved in recognizing the host signal and for transfer of T-DNA to the host cell. From Hooykaas and Beijersbergen (1994).

The transformed cells produce IAA and CK, resulting in the proliferation and growth of new cells that also carry the modified genome. Cell proliferation

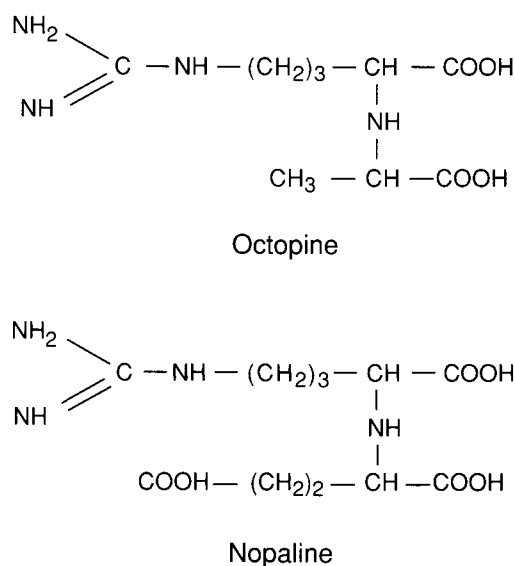


FIGURE A2-3 Structures of octopine and nopaline.

and growth create a sink for the transport of photoassimilates to the tumor, but opine synthesis enzymes, expressed in the host cells, convert the photoassimilate to opines, which are secreted out of the gall. Thus, a whole battery of plant host cells are recruited and redirected to produce nutrients for bacteria. *Agrobacterium* stay on the surface of the gall, or in the adjacent soil, and get their nourishment from the secreted opines. The elegance of the system is that enzymes for the catabolic breakdown of opines are encoded by genes that are still on the plasmid within the *Agrobacterium*. Because no other bacteria in the soil or on the gall surface have the same enzymes, *Agrobacterium* of the same strain have the monopoly on the opines produced by the host cells. Moreover, the production of opines stimulates other *Agrobacterium* that were living peacefully in the soil to acquire the Ti plasmid *via* conjugation and become virulent.

2.2. Genes Encoding Indoleacetic Acid (IAA) and Cytokinin (CK) Synthesis Enzymes

Genetic studies on *A. tumefaciens* led to an identification of three loci that determine the tumor morph-

ology. Mutations at the *tms* locus¹ (tumor morphology "shooty" or *tms* mutants) cause excessive shoot development in tumors or in callus cultures from tumor explants, whereas those at the *tmr* locus (tumor morphology "rooty," *tmr* mutants) result in a proliferation of roots.

Mutations at the *tml* locus (tumor morphology "large," *tml* mutants) cause abnormally large, unorganized tumors. These loci are present in T-DNA (Fig. A2-4).

The *tms* locus has two genes, *tms1* and *tms2*, which encode two enzymes, tryptophan monooxygenase and indoleacetamide hydrolase, respectively. These two enzymes, in a two-step process, give rise to IAA from tryptophan (Fig. A2-5). Mutations in either gene lead to a deficiency in the production of IAA, which, in turn, alters the IAA/CK ratio in the tissue. The increase in the relative concentration of CK vs that of IAA causes excessive shoot proliferation.

The identity of the proteins encoded by *tms1* and *tms2* was established by the analysis of protein extracts from strains mutated at *tms1* or *tms2*, coupled with functional expression of the two genes in *Escherichia coli*. For example, the mutant strain *tms2* fails to produce IAA, but accumulates indoleacetamide, whereas *tms1* expressed in *E. coli* gives rise to a product that is able to convert tryptophan to indoleacetamide.

The *tmr* locus carries an *ipt* gene, which encodes an isopentenyl transferase, the enzyme that catalyzes the transfer of the isopentenyl side chain from isopentenyl diphosphate (IPP) to adenosine 5'-monophosphate (5' AMP) (Fig. A2-6). The resulting product, isopentenyladenosine monophosphate, is the precursor for isopentenyladenine (IP), as well as zeatin (Z) and dihydrozeatin (diHZ). These later steps are catalyzed by enzymes encoded by the plant genome (see Fig. 8-5, Chapter 8; see also Section 4, this chapter). A mutation at the *tmr* locus leads to a deficiency in CK production and to a change in the IAA/CK balance in favor of IAA, which results in root proliferation.

Several strains of *A. tumefaciens* carry another gene for the synthesis of isopentenyladenosine monophosphate, called *tzs* (*trans*-zeatin secretion), which is located on the Ti plasmid outside of T-DNA. The *tzs* also encodes an isopentenyl transferase. Although both enzymes serve the same function, they are different

¹The nomenclature of genetic loci, mutants, wild-type genes, and encoded proteins in the literature on bacteria is different from that in *Arabidopsis*. Genetic loci and genes are italicized but referred to in lowercase, e.g., *tms* locus; a mutant at that locus is referred to as a *tms* mutant. A gene is italicized and referred to in lowercase, except part of the name may be in uppercase, e.g., *tms1*, *iaaM*, and *virA*. A protein is referred to as IAAM, or VirA, etc.

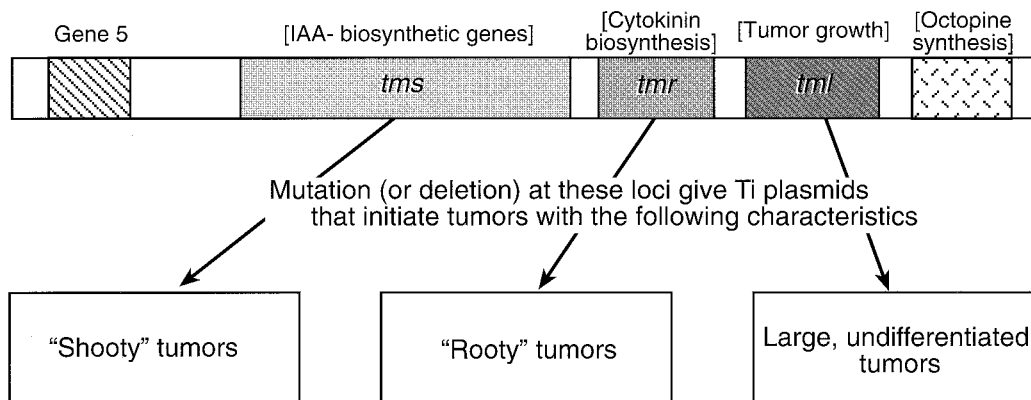


FIGURE A2-4 Map of T-DNA from an octopine Ti plasmid and the main structural features. The *tms* locus is involved in IAA biosynthesis. Mutations in that locus, i.e., *tms* mutants, give rise to tumors with a shooty phenotype. The *tmr* locus is involved in cytokinin biosynthesis. Mutations in this locus, *tmr* mutants, show an excessive proliferation of roots. The *tml* locus and gene 5 together affect the morphology of the tumor but do not cause tumorigenesis. *tml* Mutants give rise to abnormally large tumors on some hosts. Gene 5 is involved in the synthesis of indole-3-lactic acid, a biologically inactive analog of indole-3-acetic acid. Another gene encodes an octopine synthase, the enzyme responsible for the synthesis of octopine. Adapted from Morris (1986).

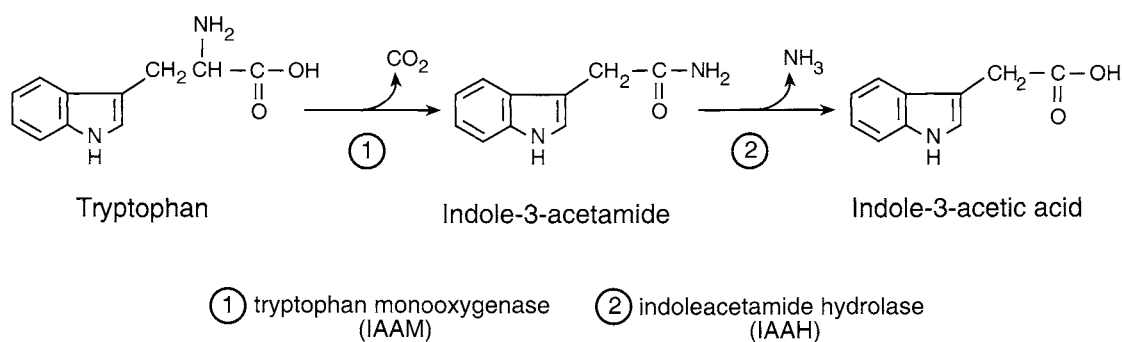


FIGURE A2-5 Microbial synthesis of IAA from tryptophan. In a two-step pathway, tryptophan is oxidatively decarboxylated to indoleacetamide, and the latter compound is deaminated to IAA. This pathway is simpler than those postulated from tryptophan for higher plants (see Fig. 6-3, Chapter 6).

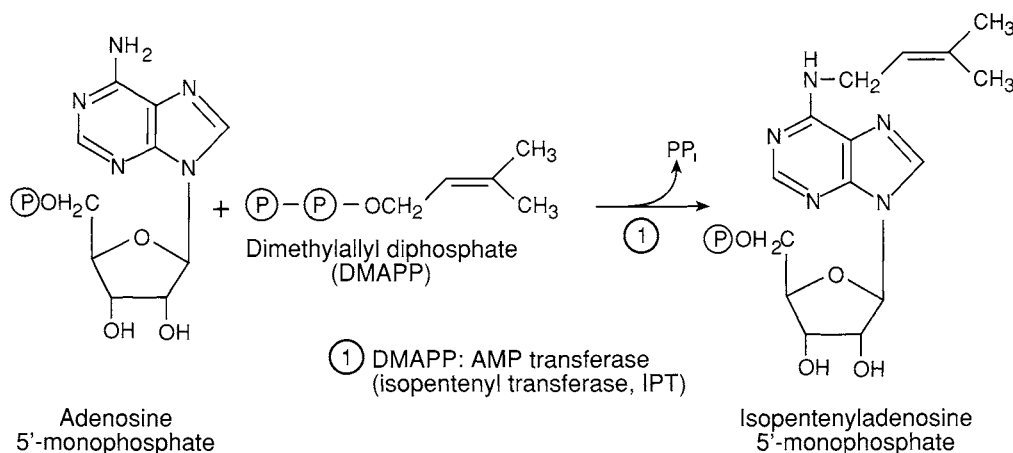


FIGURE A2-6 Microbial synthesis of cytokinin. The *ipt* gene encodes an isopentenyl transferase, which catalyzes the conversion of IPP [actually dimethylallyl diphosphate (DMAPP), an isomer of IPP; see the terpenoid pathway, Fig. 7-5] and 5'AMP into isopentenyladenosine 5'-monophosphate. The latter compound is converted to IP, Z, and diHZ by subsequent steps catalyzed by enzymes encoded by plant genes.

proteins and are regulated differently—*tzs* has a prokaryotic regulation, and *ipt* a eukaryotic regulation. It is believed that the TZS protein may be involved in inducing cell division in early stages of infection before integration of T-DNA into the host genome (see Section 6.4).

2.3. Genetic Transformation by *A. rhizogenes*

A. rhizogenes utilizes a similar strategy to that of *A. tumefaciens*. It also harbors a plasmid, known as root-inducing or Ri plasmid. T-DNA of the Ri plasmid carries a more complicated set of genes, and most of them are involved in modifying the IAA levels (see Table A2-1). Many strains (not all) of *A. rhizogenes* carry two genes, *aux1* and *aux2*, which are homologous to *tms1* and *tms2*, respectively, and are involved in IAA biosynthesis. Four other genes, called *rol* genes (root loci), *rolA*, *rolB*, *rolC*, and *rolD*, are involved in root induction or root phenotype. The precise details are unclear, but at least two of them, *rolB* and *rolC* are reported to encode enzymes that act as glucosidases that hydrolyze IAA glucosides, and CK glucosides, respectively. These *rol* genes, therefore, alter the endogenous levels of IAA and/or CK. Some strains do not carry any *aux* genes. A homologue of the *ipt* gene is usually absent from T-DNA, but a *tzs* homologue, encoding an isopentenyl transferase, is present on the Ri plasmid, outside of T-DNA.

3. TUMOR INDUCTION BY PSEUDOMONAS

As stated before, bacteria other than *Agrobacterium* that cause galls or tumors or witches' brooms on infected plants do not cause a transformation of the host cells. The bacterial chromosome or the plasmids carry the genes for IAA and/or CK biosynthesis; these hormones are produced by the bacterium and diffuse into the neighboring plant tissue, stimulating cell proliferation and growth. In these cases, therefore, the continued presence of bacteria is necessary for the maintenance of infection.

Pseudomonas savastanoi, which causes galls on olive (*Olea europaea*) and oleander (*Nerium oleander*) trees, has been most studied. This bacterium carries two genes for IAA biosynthesis, called *iaaM* and *iaaH*, which

encode tryptophan monooxygenase and indoleacetamide hydrolase, respectively. These enzymes catalyze IAA biosynthesis in the same manner as in *Agrobacterium* (see Fig. A2-5). The bacterium also carries an *ipt* gene, which encodes an isopentenyl transferase that catalyzes the synthesis of isopentenyladenosine monophosphate (see Fig. A2-6). In some strains of *P. savastanoi*, these genes are carried on a plasmid, whereas in others they may be carried on the bacterial chromosome itself. A gene homologous to *tzs*, called *ptz* (*Pseudomonas trans-zeatin*), has also been identified in *P. savastanoi* and *P. solanaraceum*, which infects members of the potato family (Solanaceae).

4. SUMMARY OF MICROBIAL GENES INVOLVED IN IAA AND CK BIOSYNTHESIS

Genes encoding IAA and CK biosynthesis enzymes in *A. tumefaciens*, *A. rhizogenes*, and *P. savastanoi* are summarized in Table A2-1. Because these genes induce tumor formation, or neoplasia and hypertrophy, by analogy with animal systems, they are referred to as "oncogenes."

There is considerable sequence similarity among *tms1*, *aux1*, and *iaaM* genes and among *tms2*, *aux2*, and *iaaH* genes, especially in their coding sequences. In view of these similarities and because they serve the same function and because the genes in *Pseudomonas* were described first, the *tms1* and *tms2* genes are now referred to as *iaaM* and *iaaH* genes, respectively; *aux1* and *aux2* should perhaps also be renamed. The *ipt* gene in *A. tumefaciens*, likewise, is homologous to the *ipt* gene in *P. savastanoi*, and both are similar to *tzs* and *ptz* genes. These similarities suggest a common origin for these microbial genes, but whether they are derived from higher plants or whether they originated first in the microbes and subsequently were incorporated in plant genome is an open question.

It should also be noted that isopentenyl transferase catalyzes the first step in cytokinin biosynthesis, i.e., the transfer of the isopentenyl side chain to adenosine monophosphate. Isopentenyladenosine monophosphate gives rise to IP, Z, diHZ, their ribosides or ribotides, or other products by subsequent steps catalyzed by plant-derived enzymes. As a result, the specific types of CKs produced, as well as their respective amounts, are determined by host cells and vary considerably from host to host.

5. EXPRESSION OF BACTERIAL GENES IN HIGHER PLANTS

The availability of bacterial genes encoding enzymes for IAA and CK biosynthesis has allowed transformation experiments, whereby the endogenous levels of these hormones can be manipulated and their effect on morphology of the plant, as well as on metabolism of the hormone in question, can be tested. Such transformations are a valuable alternative to the exogenous application of hormones or their inhibitors to plant tissues or explants.

These transformations have provided genetic evidence for the physiological observations that relate IAA concentration to apical dominance and promotion of rooting in stem cuttings or callus cultures, and CK to enhancement of lateral branching and inhibition of rooting. Thus, the introduction of *iaaM* and *iaaH* genes, or of *iaaM* singly under a strong constitutive promoter, leads to elevated levels of IAA in the transformed plant. These plants show stunting, much reduced branching, and formation of adventitious roots. In callus cultures, they show suppression of shoot growth and enhanced rooting. In contrast, the introduction of *ipt* gene leads to reduced apical dominance and a bushy habit with enhanced lateral branching, poor stem and leaf development, and an underdeveloped root system. In callus cultures, it leads to shoot proliferation and suppressed rooting.

Higher endogenous levels of IAA or CK in these transformed plants also lead to the formation of various conjugates, as well as to products associated with the irreversible breakdown of these hormones (see Chapters 6 and 8).

The *iaaL* gene of *Pseudomonas* encodes IAA lysine synthetase. IAA conjugates with lysine have not been known to occur naturally in higher plants, but *iaaL* introduced in higher plants causes increased IAA conjugation and results in a phenotype reminiscent of IAA deprivation.

6. BIOLOGY OF GENETIC TRANSFORMATION BY *A. TUMEFACIENS*

The proliferation of new host cells and their recruitment to produce opines provides *Agrobacterium* with a comfortable niche, but the machinery used by *Agrobacterium* can also be used to transform a plant. In order to understand that, we have to know how the *Agrobacterium* transfers T-DNA to the host plant. This

is done by the *virulence* (*vir*) region. The *vir* region carries genes that encode proteins that are important in the recognition of a signal from the host and in excision and transfer of T-DNA into the host cells.

6.1. Recognition of Signal

Although a few exceptions are known, in nature, only wounded or injured plants are infected. The cut surfaces exude a sap, which, depending on the species, varies in pH and compounds released by the damaged cells. An acidic pH (5.0 to 5.8) and the presence of certain types of phenolic compounds, compounds that are intermediates in lignin and flavonoid biosynthesis, e.g., acetosyringone, sinapic acid, and coniferyl alcohol, and are naturally produced, serve as signals to *Agrobacterium* (Fig. A2-7). These signals are recognized by two proteins, products of two *vir* genes (*virA* and *virG*), which act as an antenna system to sense the phenolics and to prime the other *vir* genes to produce their products.

6.2. Bacteria Are Attracted to the Host and Establish a Close Contact with the Host Cell Surface

As a result of the signal, *Agrobacterium* are also chemotactically attracted to the wound surface. For successful infection to occur, it is essential that a close contact be established between the host cell surface and an *Agrobacterium*. Bacterial chromosomal genes play no direct role in the transfer of T-DNA, but some of them are involved in the production and secretion of polysaccharides that are required for establishing this contact. Mutations in these chromosomal genes render the *Agrobacterium* avirulent.

6.3. Excision, Processing, and Transfer of T Strand to the Host Cell

T-DNA is delineated by a right (R) and a left (L) border, each about 25 bp and an imperfect copy of each other, but in a precise orientation in the Ti plasmid. For successful transformation, it is essential to maintain the sanctity and orientation of the borders, especially the R border.

The *vir* region carries several other genes, e.g., *virD* and *virB*. Some of these genes encode proteins that serve as endonucleases/helicases to cut the coding strand of the T-DNA and remove the single-stranded T-DNA, known as the ssT-strand or simply T

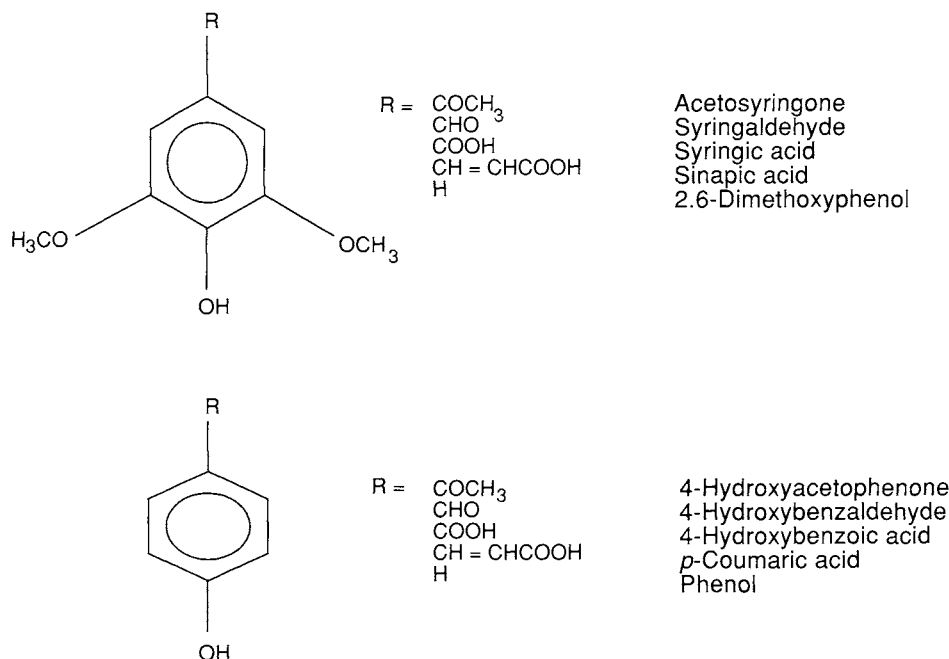


FIGURE A2-7 Structures of some phenolics that act as signal molecules for *Agrobacterium*.

strand (Fig. A2-8). The cut segment is repaired by DNA repair machinery. Other *vir* gene products protect the T strand against nuclease digestion by forming a protein coat around it and escort it out, whereas still others form a channel (pore) in the bacterial cell membrane. Some may also chaperone it through the host cell cytoplasm and direct it to the host cell nucleus.

6.4. Integration of the T Strand in the Host Genome

How the T strand gets incorporated into one of the strands of the host DNA is not fully understood. A hypothetical scheme is shown in Fig. A2-9. Variations on the scheme are known. The insertion of T-DNA into host genome occurs with very little loss of host DNA, only about 20–30 bp, and without any major upheavals. Following insertion, some trimmings and rearrangements may occur. Because only a small segment of host DNA is lost initially, it is believed that (1) only a small nick is created in the target DNA, which later widens, and (2) T strand must initially form a loop while being held in place by limited and transient base pairing. Following ligation of the T strand in the target DNA, and breakage of the other host strand, a

complementary copy is produced, giving rise to T-DNA.

T-DNA is inserted randomly in the host genome. Some data suggest that insertion occurs mostly in dividing cells or those parts of the host DNA that are transcriptionally active, which means that they are unwound and accessible. It will be recalled that the *tzs* gene, carried on the Ti plasmid outside of T-DNA, is involved in cytokinin biosynthesis, and it has been suggested that it may prime the cells for transformation by inducing cell division. The *tzs* gene does not require activation by VirA/VirG sensing proteins.

6.5. Host Specificity of *Agrobacterium* Strains

The host specificity of *agrobacterium* strains depends on the specific pH, as well as on the phenolics secreted by the host plant. Also, the infection can occur only in a “window of opportunity,” i.e., from the time of injury to the time of wound healing. This window, depending on species, varies between 12 and 96 h after wounding. Most dicots and gymnosperms are susceptible, but most legumes and monocots, especially members of the grass family, are immune to tumorization by *Agrobacterium* in nature (but see Box

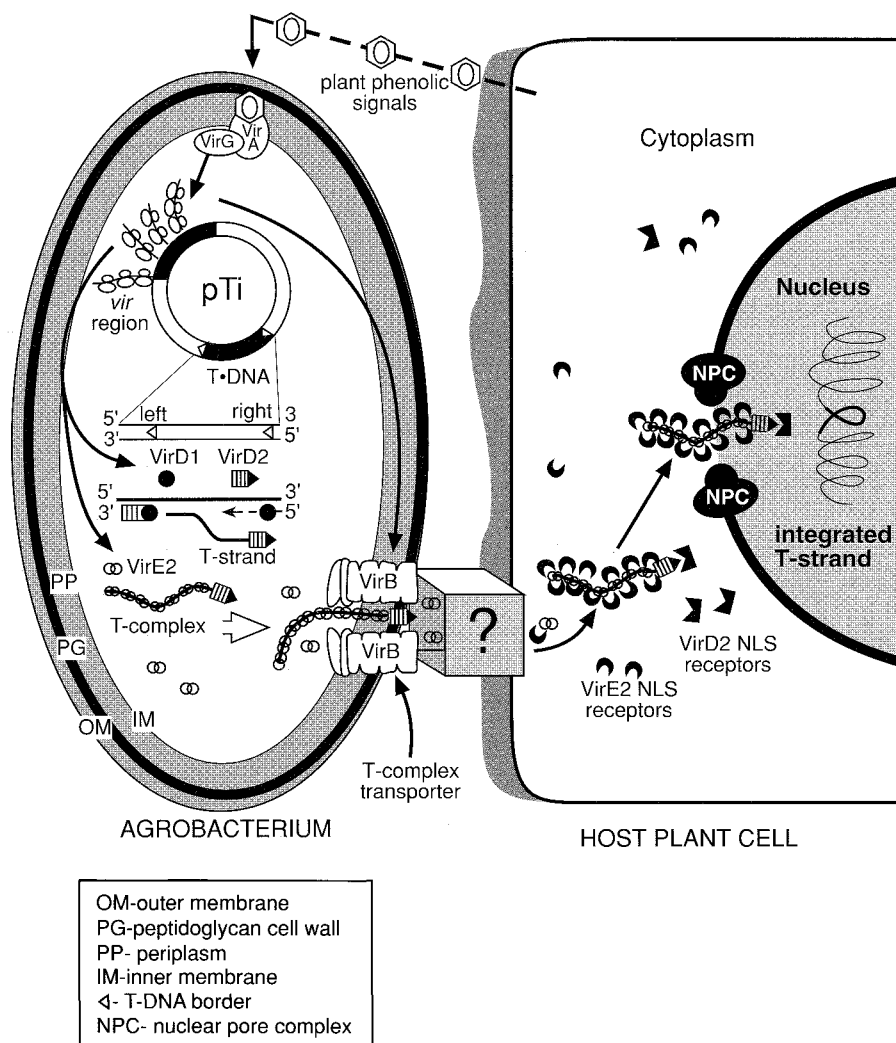


FIGURE A2-8 Summary diagram showing excision of the T strand and its transfer to the plant cell. Many Vir proteins are involved in T-DNA excision, its processing, transfer to the host nucleus, and integration into the host DNA. VirA and VirG proteins recognize the plant phenolic signals and activate the transcription of other *vir* genes. VirD1 and VirD2 are involved in the nicking and helicase reactions that give rise to the T strand; VirD2 also binds covalently to the 5' end of the T strand and serves as a pilot protein to guide the T strand from the bacterium to the host nucleus. VirE2 forms a protective coat around the T strand/VirD2, now known as the T complex, as shown, although it is possible that such an association occurs only after independent entry into the host cell. The VirB protein(s) forms a pilus on the bacterial surface through which T-DNA and the transferred proteins move out of the bacterium and into the plant cell. VirD2 and VirE2 both carry plant-specific nuclear localization signals (NLS) and, in association with host cytoplasmic factors, are thought to help nuclear entry and integration of T-DNA into plant DNA. With permission from Zupan *et al.* (2000), ©2000 Blackwell Science.

A2-1 below for the transformation of agricultural crops, such as maize and rice). Why these plants are not infected in nature may be related to the specific compounds released on injury, as well as on the specific wound response, which affects the window of opportunity. Plants may not produce a compound that is specifically recognized by the sensing machin-

ery of a particular strain of *Agrobacterium*, but the strain may metabolize it to a compound that is recognized. Thus, Douglas fir (*Pseudotsuga menziesii*) secretes coniferin, which is not recognized by *Agrobacteria*, but certain strains of *Agrobacteria* produce sufficient amounts of a β -glucosidase that hydrolyzes it to coniferyl alcohol, which is recognized.

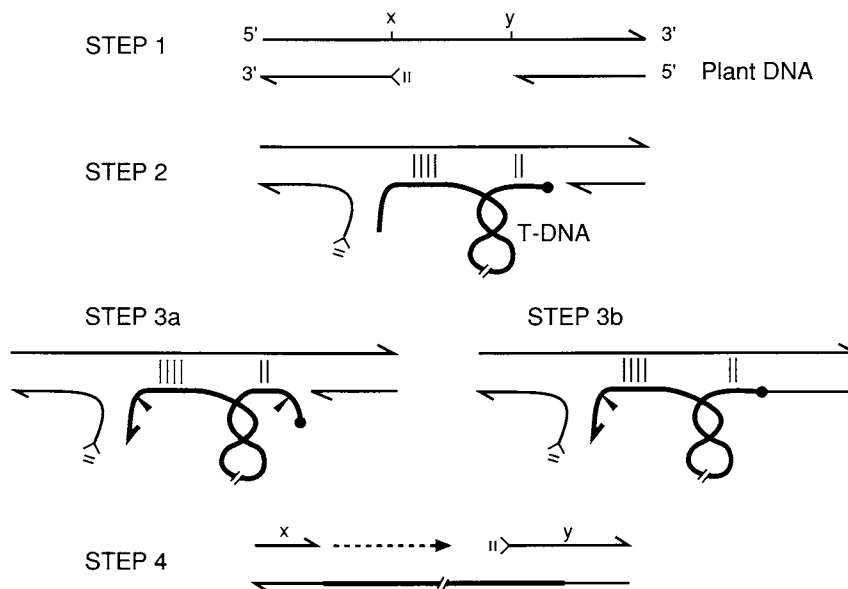


FIGURE A2-9 A model showing steps in the integration of T-DNA into host DNA. In step 1, a nick is created in one of the strands (the target strand) of host DNA, which widens to a gap by unwinding, as well as by 5' → 3' exonuclease activity. In step 2, the T strand invades the gap and is held in place by limited base pairing (thin vertical bars) between the other host strand and the ends of the T strand. In steps 3a and 3b, the overhanging ends get nicked off and removed, and the T strand gets ligated. In step 4, torsional strain on the other host DNA strand causes it to break and be repaired by normal DNA repair machinery, producing a complementary copy of the T strand. The size of the small plant DNA deletion (x-y) associated with T-DNA integration depends on 5' → 3' exonuclease activity in step 1 and repair in steps 3 and 4. With permission from Gheysen *et al.* (1991), ©1991 Cold Spring Harbor Laboratory Press.

BOX A2-1 PLANT TRANSFORMATION

THERE ARE TWO MAIN types of transformations: transient or stable. In “transient” transformations, the introduced gene is expressed without being passed on from cell to cell. They last only as long as the transformed cell/tissue lasts and are used by researchers for monitoring special effects, e.g., production of a specific protein by the transformed cell. In contrast, “stable” transformation means that the transformation is carried from one generation to the next. Transient transformations can be accomplished very quickly and usually take much less time and effort than stable transformations.

Transient transformations Transient transformations can be accomplished using any one of several physicochemical means for inserting foreign DNA into the cell.

Chemical Methods The DNA construct can be loaded into liposomes or even left naked in solution, both can be picked up by endocytosis by plant cells; however, for this to occur, protoplasts have to be prepared by enzymatic removal of the cell wall by mixtures of cellulases and pectinases. Uptake is facilitated by chemicals, such as long chain polycations, including polyamines (e.g., poly-L-ornithine, poly-L-lysine) or charged carbohydrate polymers (e.g., dextran sulfate). Because both the DNA and the plant cell membrane are negatively charged, these charged molecules facilitate transfer. Other chemicals used are the long chain hydrophobic polymer, polyethylene glycol (PEG).

Electroporation Electrical impulses induce transient pores in the plasma membrane (PM). The pores can be up to about 30 μm in diameter and persist for several minutes after the pulse, which is long enough to permit the movement of molecules across the PM into the cell. The two main factors are the voltage

gradient and the pulse duration (or the decay time). Protoplasts are placed in an ionic solution, which contains the DNA construct. The mixture, in a suitable container, is suspended between two electrodes across which a discharge occurs (~ 1.5 kV for 10 μ s); the whole thing is carried out in a specially designed electroporation chamber. PEG can also be used.

Microinjection DNA constructs may be microinjected into the cytoplasm, or nucleus, of cells or protoplasts that have been immobilized on an agarose gel or polylysine beforehand. The injection is usually performed under an inverted microscope with a glass pipette, which has been drawn into a fine syringe $\sim 0.5 - 10$ μ m in diameter.

Ballistic Gun That Shoots Microprojectiles Ballistic guns have been devised that shoot microprojectiles. Tungsten or gold microprojectiles are coated with the DNA construct, spread on a plastic or nylon bullet, and shot with a ballistic gun onto a plate that stops the bullet, but allows the spread of tungsten or gold microprojectiles into the plant tissue. The shock wave is caused by vaporizing a water droplet or by a cordite explosion. This method may be used with whole tissues.

As mentioned earlier, these methods are good for transient transformation, but they can also give stable transformation if the transformed cell(s) (e.g., protoplast, pollen, or callus tissue) can be used to generate a whole plant using embryo or tissue culture techniques.

Stable transformation using *Agrobacterium* Transformation of plant cells using *Agrobacterium* has three main advantages over transient transformation: (i) it provides for stable transformation; (ii) it allows insertion of one to several copies of the gene of interest into the plant genome; and (iii) it allows a high coexpression of the introduced genes. Transformation of a plant tissue using *Agrobacterium* is usually more complicated than physicochemical means of DNA delivery, especially in earlier steps, because it involves preparation of a plasmid construct.

As we have seen, T-DNA is flanked by two 25-bp borders, a R and a L border. These borders, especially the R border, which also has to be in correct orientation, are very important for the correct excision and transport of the T strand through the bacterial to the host cell by the various Vir proteins. The part of T-DNA between the borders, its sequence or orientation, is of little consequence. In theory, it can be completely removed and replaced with the gene of interest, henceforth called the "foreign" gene, and the *agrobacterial* Vir machinery will still excise and transfer it as if it were the original T strand. In actual practice, things are more complicated. Several steps are involved.

Preparation of the Plasmid Construct The Ti plasmid is quite large and it is difficult to manage a correct insertion of the foreign gene between the borders. Therefore, use is made of a smaller surrogate plasmid, e.g., from *E. coli*. These plasmids have to be such that they are compatible with the particular strain of *Agrobacterium* being used, i.e., they can multiply with the bacterium in culture. The foreign gene ligated between the R and the L borders is inserted in this surrogate plasmid. Because the Ti plasmid still has to be used for its virulence machinery, the Ti plasmid is "disarmed," i.e., the segment of T-DNA containing the hormone and opine synthesis genes is removed. Thus, the engineered *Agrobacterium* has a disarmed Ti plasmid with its vir machinery intact and a surrogate plasmid with the foreign gene between the R and the L borders in correct orientation. These engineered bacteria are allowed to multiply in culture.

Selection of Transformed Cells/Tissue In a cell mass or tissue, it is important to be able to recognize which cells have actually been transformed and carry the foreign gene. Because the IAA, CK, and opine synthesis genes are eliminated, there is no precise way to know that a host cell has been transformed. Accordingly, use is made of "marker genes," which are inserted along with the foreign gene. Marker genes are of two types:

- i. Selectable genes encode a product that confers resistance to an antibiotic or to a herbicide. The host cells/tissue in culture is exposed to the antibiotic or herbicide, and cells that show resistance and survive are the ones that have been transformed (Table A2-2).

TABLE A2-2 Some Commonly Used Marker Genes^a

Enzyme encoded		
Selectable genes		Selective agent(s)
<i>hpt</i> (antibiotic)	Hygromycin phosphotransferase	Hygromycin B
<i>npt II</i> (antibiotic)	Neomycin phosphotransferase	Kanamycin, Neomycin
<i>aro A</i> (herbicide)	5-Enolpyruvylshikimate-3-phosphate synthase	Glyphosate (Roundup)
Reporter genes		Substrate(s) and assays
<i>CAT</i>	Chloramphenicol acetyl transferase	[¹⁴ C] Chloramphenicol and acetyl-CoA
<i>GUS</i>	β-Glucuronidase	Range of substrates depending on assay. Colorimetric, fluorometric, and histochemical techniques are available
<i>lux</i>	Luciferase	Decanal and FMNH ₂ , or ATP, O ₂ , and luciferin

^aFrom Draper and Scott (1991).

ii. Reporter or screening genes encode an enzyme whose presence can be visualized by a chemical reaction. Genes encoding the enzymes are usually microbial and not present in plant cells.

Generation of Transformed Plants Plant tissues used for transformation (e.g., leaf or stem explants, callus tissue, or protoplasts) are exposed to engineered *Agrobacteria* that carry the foreign and marker genes. Signaling compounds, such as acetosyringone, may be provided and the plant tissue is wounded or disturbed, although these steps are not essential.

The transformed plant cells are selected by either of the two means mentioned earlier, separated from the untransformed tissue/cells, and whole plants are regenerated. The plants can be regenerated from callus tissue either by following the protocol for somatic embryogenesis or by inducing root and shoot formation in callus tissue and subsequent transplantation.

The insertion of the foreign gene is random. The construct itself may get cut up or get inserted in different orientations, but as long as the foreign gene is expressed, the transformation is a success.

Using these techniques, many dicots, including agronomically important crops, such as soybean, cotton, peanut, and pea, were genetically transformed in the 1980s. The monocots proved recalcitrant for many years. However, starting in the early 1990s, many crop plants, including maize, rice, and wheat, have been transformed with genes of interest using *Agrobacterium*. In many of these transformations, embryo tissues or embryo-derived calli have been used as the source material.

7. PRODUCTION OF PLANT HORMONES BY OTHER MICROORGANISMS

Several other bacteria, fungi, and slime molds produce and secrete plant hormones (see Table A2-3). Among these, the great majority produce IAA and/or CKs. Many of these reports stem from the identification of cytokinins and/or IAA in culture filtrates of bacteria or fungi and, in some cases, where galls or leaf nodules are formed, by analysis of plant tissue. The role of these hormones in the symbiotic association and/or in the pathogenic process is not clear. Patho-

genic strains of *Erwinia herbicola* harbor a plasmid, which carries genes for IAA and CK biosynthesis. However, the IAA synthesis in this case is reported to occur from tryptophan *via* indolepyruvic acid, the same pathway used by higher plants for tryptophan-derived IAA (see Chapter 6). The *bam* gene from *Bradyrhizobium* was isolated and sequenced and has homologies with the *iaaH* gene. In most cases, the genes responsible for synthesis have not been identified and few molecular data are available on these microorganisms. Some microbes listed in Table A2-3 cause gall/tumor formation or other abnormal growth, whereas others do not show any visible hyperplasia. Table A2-3

TABLE A2-3 A Partial Listing of Microbes and Insects That Produce Plant Hormones

	Association type	Hormone(s)	Refs.
Bacteria			
<i>Pseudomonas amygdali</i>	Almond canker	IAA, CKs	Morris (1995)
<i>P. solanacearum</i>	Bacterial wilt	CKs	Morris (1995)
<i>Erwinia herbicola</i> pv <i>gypsophillae</i>	Gypsophila gall	IAA, CKs	Morris (1995), Nizan <i>et al.</i> (1997)
<i>Rhodococcus fascians</i> (<i>Corynebacterium fascians</i>)	Witches broom disease	CKs	Morris (1995)
<i>Azotobacter</i> sp.	Rhizosphere	CKs	Morris (1995)
<i>Arthrobacter</i> sp.	Rhizosphere	CKs	Morris (1986)
<i>Azospirillum</i> sp.	Rhizosphere	IAA	Morris (1995)
<i>Rhizobium</i> sp.	Symbiont	CKs	Morris (1995)
<i>Bradyrhizobium japonicum</i>	Symbiont	CKs	Morris (1995)
<i>Frankia</i> sp.	Symbiont	CKs	
<i>Chromobacterium lividum</i>	Leaf nodule endophyte	CKs	Morris (1986)
Fungi			
<i>Rhizopogon roseolus</i> , <i>Boletus edulis</i>	Ectomycorrhizae	CKs	Ng <i>et al.</i> (1982)
<i>Helminthosporium</i> sp. <i>Ustilago</i> sp. (rusts and mildews)	Green islands on leaves	CKs	Morris (1986)
<i>Gibberella fujikuroi</i>	Pathogen	GAs, JAs	Graebe (1987)
<i>Sphaceloma manihoticola</i>	Pathogen	GAs	Graebe (1987)
<i>Cercospora rosicola</i>	Rose spot	ABA	Bennett <i>et al.</i> (1990)
<i>Fusarium oxysporum</i>	Pathogen	JAs	Miersch <i>et al.</i> (1999)
Slime mold			
<i>Dictyostelium</i>	Free living	CKs	Morris (1986)
Insects			
Wasp larvae	Galls in <i>Erythrina</i> <i>latissima</i>	CKs	Morris (1986)

also lists some fungi that produce cytokinins in a symbiotic relationship with plant roots (mycorrhiza) or a pathogenic relationship with higher plants in association with roots. Many other fungi produce gibberellins, abscisic acid, or jasmonates. Some bizarre examples of insect larvae that feed on leaves and cause galls, presumably by producing cytokinins, are also known.

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III

HORMONAL REGULATION OF DEVELOPMENTAL AND PHYSIOLOGICAL PROCESSES

...many—perhaps all—growth phenomena are the result of interaction and balance between several hormones,... (Kenneth Thimann, 1977, p. 203)

All plant hormones have multiple roles in plant growth or defense, i.e., they are pleiotropic in their effects. To this basic theme, a layer of complexity is added by the fact that two or more hormones bring about the same, or similar, responses. For example, cell division is mediated by cytokinins (CKs), auxins, or gibberellins (GAs). Cell enlargement involves action by auxin, GA, brassinosteroids (BRs), or ethylene. Absciscic acid (ABA), ethylene, and jasmonates (JAs) are involved in the ability of a plant to cope with biotic or abiotic stresses. This redundancy is a hallmark of plant development, although it is not clear whether it is real or only apparent, i.e., two hormones regulate closely related but different aspects of the same process.

Plant hormones also regulate some activities that are specific to each hormone. For instance, patterning in embryo development and polar phenomena such as apical dominance, vascular differentiation, and tropic growth under the influence of light or gravity are principally regulated by the endogenous auxin, indoleacetic acid or IAA; mobilization of seed food reserves in cereal grains is specific to GAs; seed dormancy is induced by ABA; fruit ripening is associated with ethylene; and JAs are uniquely involved in the deposition of vegetative storage proteins.

The chapters in Section III of this book reflect the apparent redundancy and uniqueness of hormone action. They also show that even relatively simple processes,

such as apical dominance or production of lateral roots, involve an interaction of two or more hormones and their relative levels, which are affected by environmental and/or developmental cues. Such interaction is often antagonistic, as, for example, IAA and CK interaction in lateral root formation, although it is not clear whether such antagonism is used for regulation of the process in nature. Synergistic interaction also occurs, e.g., for ethylene and JA in induction of some genes in plant defense against pathogens. Finally, several hormones may act in concert, one after another, to regulate a sequence of developmental events. For example, fruit set may be regulated by IAA, fruit growth by GA, fruit ripening by ethylene, and seed maturation and dormancy by ABA. Because of these interactions among hormones and between hormones and environmental factors, the extent of which we have only recently begun to appreciate, an understanding of plant hormonal response is a complex and difficult fabric to unentangle.

In the past, plant hormones have been credited with a bewildering array of responses—there is hardly any plant process that has not been attributed to one or another hormone. Such conclusions were often drawn from treatment of whole plant or isolated organs/tissues with exogenous hormones, often at unnaturally high concentrations. These treatments disturb the natural homeostasis and generally do not provide a true indication of the role of a hormone. That role is deciphered better by the use of mutants that are deficient in or are insensitive to a specific hormone or by a defined biochemical response to a hormone. For this reason, not all responses attributed to hormones are covered in these chapters; rather the chapters are selective in that they deal with only the better understood and, hopefully, major responses and processes.

The uptake and transport of hormones in the plant body are covered first in Chapter 13; Chapter 14 deals with IAA and CK interaction and plant responses related to the polar transport of IAA (some others are covered in Section V of this book). Hormonal regulation of cell division and cell enlargement, both important for plant growth, are covered in Chapter 15. Chapter 16 deals with the mechanisms plants use to cope with the main types of abiotic stresses and the role of ABA in induction of those mechanisms. Not surprisingly, hormones that are involved in growth-related processes play a minimal role in stress-related responses, and *vice versa*. Fruit and seed development and seed germination, covered in Chapters 17, 18, and 19, are growth-related processes in which CKs, IAA, and GAs play important although still little understood roles, whereas fruit ripening and seed maturation and dormancy are culminating phases of growth, akin to senescence, and are regulated by ethylene, ABA, and possibly JAs. Chapter 20 covers several plant processes, accumulation and role of vegetative storage proteins, tuber formation in potato, senescence and abscission of plant parts and organs, and the roles of various hormones in these processes. Finally, Appendix 3 describes the major food reserves and their accumulation in seeds.

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13

Uptake and Transport of Hormones

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1. INTRODUCTION

Hormones are taken up from the external medium by plant tissues. They are also translocated short or long distances in the plant from their sites of synthesis and/or application. This chapter will first consider the characteristics of hormone uptake and accumulation, then their long-distance translocation in vascular tissues, and finally the polar transport of auxins, which underlies many developmental phenomena in plants as well as their tropic responses to environmental signals.

2. CHARACTERISTICS OF UPTAKE AND ACCUMULATION

Plant hormones are small molecules, which can easily diffuse across cell boundaries in response to their concentration gradient. However, such movement is governed by a complex interaction of several factors, and how uptake occurs in a specific case *in vivo* is still not fully understood. This section outlines some general concepts on hormone uptake by diffusion and *via* specific carriers, followed by a discussion on the auxin influx carrier.

Auxins, gibberellins (GAs), abscisic acid (ABA), and jasmonic acid (JA) are weak acids, which in solution dissociate into anions and protons (Fig. 13-1). The pK_a values of IAA, ABA, and JA measured at 20°C are about 4.7–4.8; the pK_a of GA₁ is lower, about 3.85. Accordingly, at neutral pH in a free solution, these hormones exist predominantly in a dissociated form, but at pH closer to the pK_a or below it, the proportion of the undissociated form increases. The situation

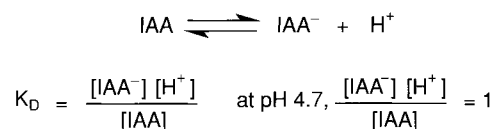


FIGURE 13-1 Dissociation of IAA. IAA in solution dissociates into IAA[−] and H⁺. The equilibrium constant (K_D) is given by the ratio of the concentration of free ions divided by the concentration of the undissociated molecule. At pH 4.7, the pK_a , the proportion of dissociated and undissociated IAA, is equal.

in planta, however, is not known. Cytokinins (CKs) and brassinosteroids (BRs) occur predominantly as undissociated molecules. Ethylene is nonpolar and gaseous and freely permeable, although its precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), is a weak acid.

2.1. Uptake by Diffusion

Undissociated hormones are freely permeable across the plasmalemma. Their movement is governed by their concentration gradient across the membrane, as well as by the size and polarity of the permeating molecule. For example, undissociated jasmonic acid, which is more lipophilic, is more permeable than undissociated ABA. Substituted derivatives of hormones may be more or less permeable than the parent molecule depending on the nature and size of the substitution. Thus, addition of a nonpolar moiety to a GA is likely to render the derivative more permeable than the parent GA, but beyond a certain size, the substitution may become an impediment to permeability. For example, the higher biological activity of 2,2'-dimethyl GA₄ than GA₄ is thought to be due to its greater permeability, but 2,2'-dibutyl GA₄ could be less permeable than the parent GA₄ because of its much larger size.

Movement by diffusion continues until the concentration of the free hormone on the two sides of the membrane is equal. Under certain conditions, however, large amounts of the hormone, albeit in a different molecular form, may be accumulated.

At mildly acidic pH (5.0–6.5), substantial amounts of IAA, GA, ABA, and JA occur as undissociated molecules and can diffuse across the plasmalemma into the cell. Inside the cell, they dissociate into their ionic forms and H⁺, and ions being charged cannot diffuse out. Since each molecular species diffuses across the membrane according to its own concentration gradient, movement of the undissociated species continues and large amounts of the dissociated hormone can be accumulated in the cytosol, a phenomenon referred to as "ion trapping" (Fig. 13-2A).

Also, the undissociated hormone may be metabolized to a different molecular species (Fig. 13-2B). For example, GA₁ may be converted to its glucosyl ester or to an inactivated derivative, such as GA₈. Under these conditions, the cytosolic levels of free GA₁ remain low, and uptake of the hormone may continue for a considerable time without reaching an apparent saturation (Fig. 13-2C). The hormone may also bind to an intracellular macromolecule, e.g., a receptor. Once bound to the receptor, it is no longer

part of the free pool in the cytosol, and diffusion of the hormone into the cell continues until the level of free hormone inside the cell is equal to that outside the cell.

2.2. Carrier-Mediated Uptake

Ions and charged or polar molecules are unable to cross artificial lipid bilayers. Nonetheless, they are able to permeate biological membranes because special transport proteins facilitate their movement across the membrane. These transporters include channels, carriers, and pumps. For transport of solutes across biological membranes, the reader is referred to texts on plant or cell physiology. A basic description is provided in Box 13-1.

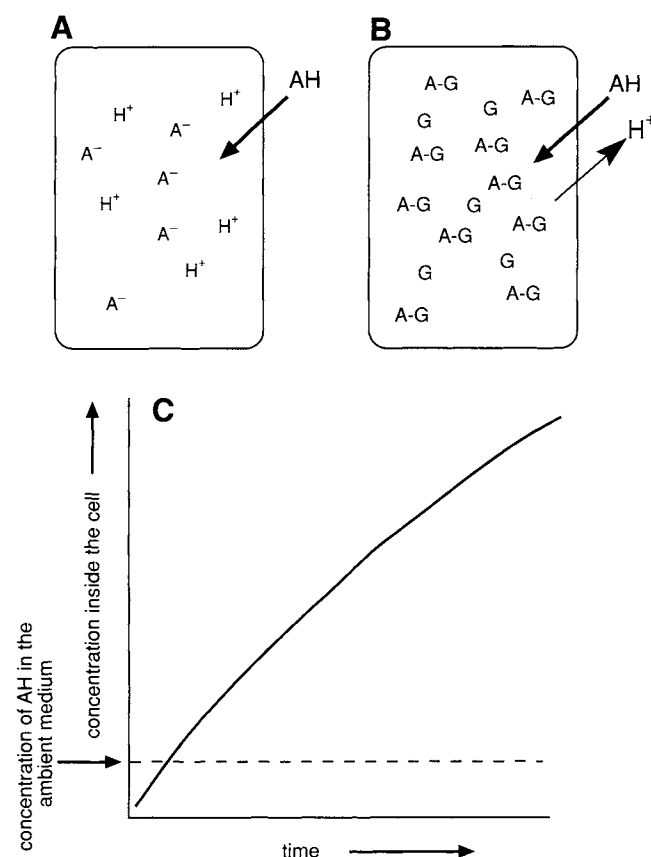


FIGURE 13-2 Accumulation of a hormone by ion trapping or metabolism. (A) The undissociated hormone (AH) continues to move into the cell; because, on entry, it is converted to its dissociated ions, A⁻ + H⁺. (B) On entry, AH is converted to its glucosyl ester, A-G, while H⁺ is pumped out of the cell. (C) Under either condition, uptake of AH into the cell continues without reaching an apparent saturation. The concentration of AH in the incubation medium is indicated for comparison.

BOX 13-1 PROTON ATPASES PLAY IMPORTANT ROLES IN CELL PHYSIOLOGY

THE TRANSFER OF IONS, both inorganic and organic, and molecules, even water, across cell membranes involves the activities of channels, carriers, and pumps. All are integral membrane proteins or protein complexes.

Channels are composed of proteins that form cylindrical passageways filled with water through which ions move across a lipid membrane. Channels exhibit some selectivity toward ionic species, with selectivity being determined by the density of the surface charge on the lining of the channel and the size of the pore. Some channels are specific to an ion (e.g., K^+ channels), whereas others are less specific (e.g., anion channels, or K^+ / Ca^{2+} channels). Channels regulate the flow of ions by opening or closing, a phenomenon known as "gating." Some channels open in response to a chemical (ligand), which binds to its receptor, usually the channel protein itself; they are "ligand-gated" or receptor-activated channels. Others respond to voltage differences on the two sides of the plasma membrane or the tonoplast—these are voltage-activated channels. Still others are opened as a result of membrane stretching, they are stretch-activated channels. The flow of ions through an open channel is passive and does not require energy. It occurs in response to a concentration gradient of the ion in question on the two sides of the membrane; but the flux of the ion through a single channel can be extremely high, frequently in excess of $10^7 s^{-1}$ and approaching the limit set by free diffusion through water.

Carrier proteins transfer an ion or a molecule from one side of a membrane to the other. They are specific to each ion or molecular species. While the movement of the ion or molecule itself is passive, the motive force for the carrier protein is provided by the proton or pH gradient and/or electrical gradient, which are set up by the proton pumps (see below). Carrier proteins may act as "symporters," transferring two different species across the membrane in the same direction, or as "antiporters," which transfer one species in one direction and the other in the opposite direction.

Pumps involve expenditure of energy and are usually coupled to an ATPase, although couplings to other energy sources also occur. Proton pumps actively secrete H^+ out of the cytosol into the apoplast and use the energy of hydrolysis of ATP to do so. They are H^+ -ATPases. The activity of H^+ -ATPases has two immediate consequences. It lowers the pH of the apoplast relative to that of the cytoplasm, resulting in a pH gradient between these two compartments. In resting cells, the pH of the apoplast varies between pH 5.0 and 6.5 compared to the cytoplasmic pH, which tends to be ~ 7.0 . Extrusion of H^+ from the cytoplasm also results in an electrical potential on the cytoplasmic side of the plasma membrane, which is negative relative to that on the extracellular side of the plasma membrane. In resting cells, the potential difference may be as negative as -100 to -150 mV. Both the pH gradient and the electrical gradient provide important motive forces for many cellular activities, including the carrier-mediated uptake of ions, sugars, amino acids, and hormones. Plant cells expend considerable amount of energy in maintaining their proton pumps (Fig. 13-3).

Other pumps use ATP energy to pump anions such as Cl^- or cations such as Ca^{2+} , K^+ or Na^+ . Pumps can be electrogenic or electroneutral. They are electrogenic if, in the process of transfer of an ion(s), they create an electrical or charge imbalance on the two sides of the membrane. A proton ATPase is an electrogenic pump; its activity results in the interior of the cell being more electronegative with respect to outside. In contrast, a pump is electroneutral, if the ions moved do not create an electrical imbalance.

Purified vesicles derived from plasma or vacuolar membranes, or membrane fractions from organelles, such as mitochondria and Golgi, are frequently used for *in vitro* experiments on the activities of transport proteins. Vesicles represent a powerful experimental system for the study of transport phenomena because the problems associated with intact cells and tissues, such as trapping of solutes in intracellular compartments or diffusion across cells, are avoided. In preparation of vesicles, care is taken that the vesicle membrane is right side out to ensure the correct orientation of the transport proteins. Specific transport proteins can also be introduced in vesicles made of artificial lipid bilayers to study their activities *in vitro*.

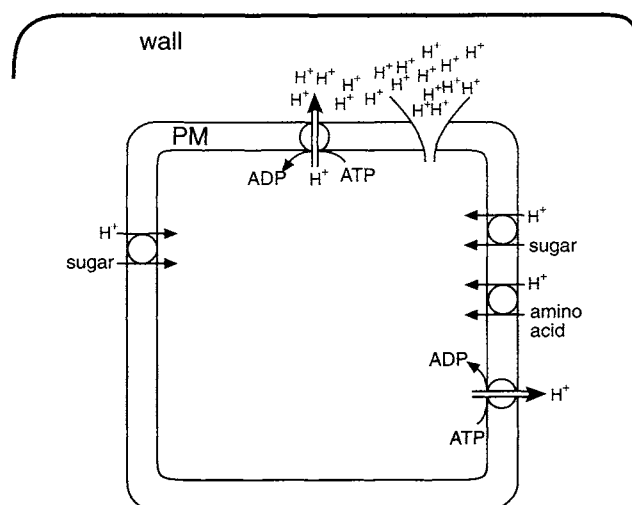


FIGURE 13-3 Coupling of ion, sugar, and amino acid transport to the proton motive force. H^+ -ATPases pump out H^+ to the exterior of the cell and, in the process, set up an electrochemical gradient, the proton motive force. The free energy thus stored is used to drive the transport of ions, sugars, amino acids, or hormones against their electrochemical gradient.

The transport of hormone ions across cell membranes occurs mostly by carriers coupled to proton motive force generated by the activity of proton ATPases. However, *in vitro*, it is affected by other factors as well, such as ionic concentrations in the apoplast and symplast, and membrane potential at the plasmalemma. Among hormones, carrier-mediated uptake has been studied mostly for auxins and, to some extent, for ABA. This topic is discussed below.

2.2.1. Demonstration of Carrier-Mediated Uptake

A carrier-mediated uptake of hormones is usually demonstrated by what is known as "saturation kinetics." If cut segments of stem or root, or suspension cells in culture, are incubated with radiolabeled hormone at a defined pH and the rates of uptake are plotted, then at some point, as all carrier molecules get saturated, a saturation in uptake rate should be reached. As explained earlier, such saturation may not be reached, however, because of hormone metabolism or binding to nonspecific macromolecules. To minimize the effects of such nonspecific binding, the plant material is also incubated in a mixture of the same amount of radiolabeled hormone plus a large excess of unlabeled hormone. The unlabeled hormone, theoretically, saturates all the nonspecific binding sites. The difference between the amount of radiolabel taken up in the two incubations gives the amount of saturable component or the labeled hormone specifically bound. (The protocol is similar to hormone-binding assays, which are explained in Appendix 4.) Data

for one such experiment for ABA uptake are shown in Fig. 13-4. As can be seen, the uptake rate of the saturable component rises sharply at first and then plateaus at the maximal rate.

2.2.2. Uptake by Membrane Vesicles

Uptake experiments can also be carried out using microsomal vesicles prepared from membrane fractions from plant tissues, with care being taken that they are sealed and are right side out, i.e., they mimic the transmembrane pH and electrical gradients that exist *in vivo*. Such vesicles from zucchini (*Cucurbita pepo*) hypocotyls have been used to demonstrate the uptake of ^{14}C -IAA from the ambient medium. *In vitro* experiments with vesicles are useful because they can be used to distinguish the effects of agents that disrupt the proton gradient without affecting the electrical gradient, or *vice versa*. Such experiments have shown that the accumulation of labeled IAA is governed primarily by the proton gradient on the two sides of the vesicle membrane, provided the inside of the membrane is at a negative potential. For IAA, it has been further postulated that H^+ -ATPase acts as a symport for entry of $IAAH/H^+$, but that a $2H^+/IAA^-$ symport is a more likely mechanism (Fig. 13-5).

2.2.3. pH Dependence and Specificity

Carrier-mediated uptake of both IAA and ABA has pH optima between pH 4.5 and 6.0 (Fig. 13-6), a pH range where both dissociated and undissociated forms

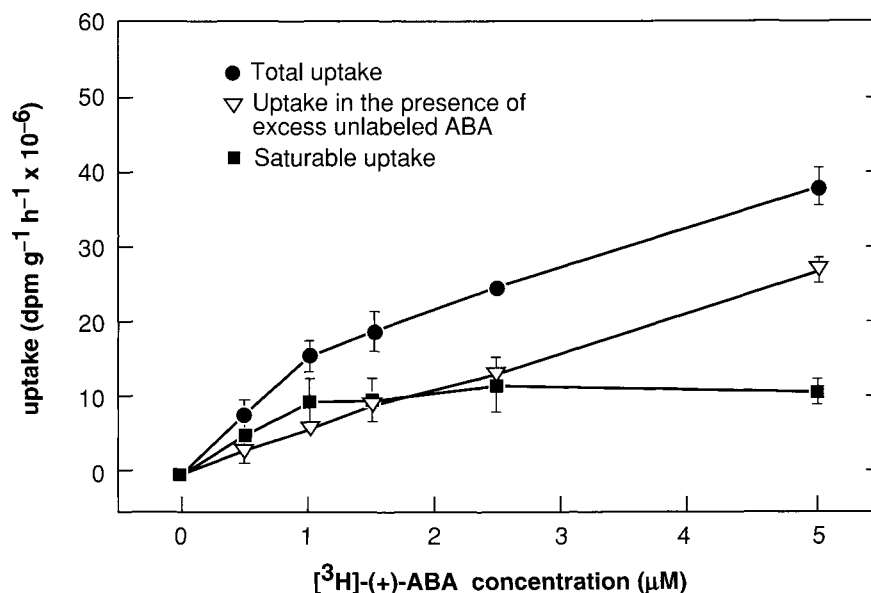


FIGURE 13-4 Carrier-mediated uptake of ABA. Barley (*Hordeum vulgare*) cells in culture were incubated in [³H]-(+)-ABA (final concentration from 0 to 5.0 μM, 10 min incubation) in buffer at pH 4.5. Rates of uptake of radiolabeled ABA were measured in the absence (total uptake) or in the presence of a large excess of unlabeled ABA (50 μM). Carrier-mediated uptake, or saturable uptake, is calculated by subtracting the radiolabel taken up in the presence of excess unlabeled ABA from that taken up in its absence (the total uptake). From Perras *et al.* (1994).

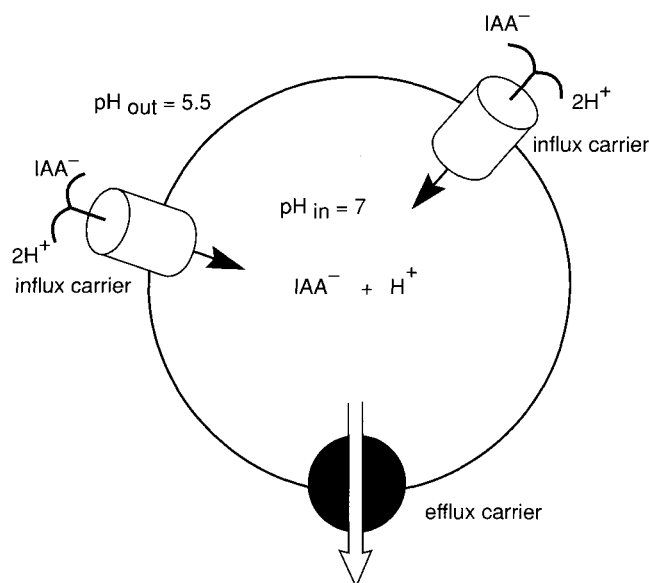


FIGURE 13-5 A model of auxin transport in plasma membrane vesicles from shoot tissues. The vesicles are isolated such that the pH and electrical potential gradients are similar to those of intact cells. Such vesicles isolated from zucchini (*Cucurbita pepo*) hypocotyls can maintain a pH gradient for many hours. These vesicles contain an electrogenic uptake symport, which transports IAA along with two protons. The vesicle is also shown with an efflux carrier. Both influx and efflux carriers are important for the polar transport of IAA in plants (see Section 4). From Lomax *et al.* (1995) with kind permission from Kluwer.

of IAA and ABA occur. Above pH 6.0, no carrier-mediated uptake is observed. The situation *in vivo* and *in planta* is not known.

Influx carriers for auxins or ABA are specific for those hormones. For example, IAA uptake by the auxin-specific carrier can be competed for by 2,4-D, but not by inactive auxins or other hormones, such as gibberellins or ABA. However, within each class of

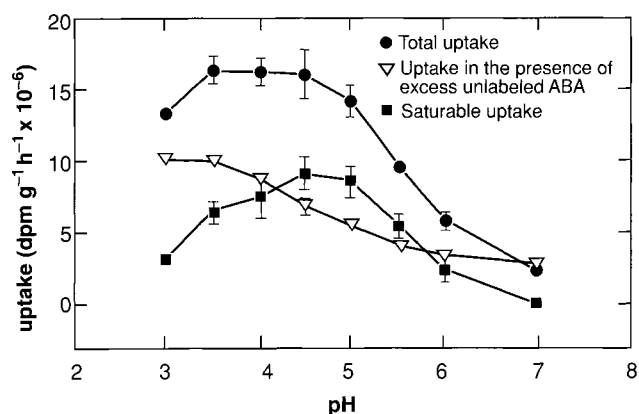


FIGURE 13-6 pH dependence of carrier-mediated uptake of ABA. Note that saturable uptake (i.e., total uptake of radiolabeled ABA minus uptake in the presence of a 100-fold excess of non-radioactive ABA) declines both toward lower and higher pH. From Perras *et al.* (1994).

hormones, there seems to be a wide latitude of structures that can be accepted by the influx carrier. Thus, the influx carrier for auxins seems to accept IAA and 2,4-D, two quite dissimilar molecules. 1-naphthalene acetic acid (1-NAA) is highly lipophilic and enters cells mostly by passive diffusion. For ABA, not only the natural enantiomer, but also several structural ring modifications are accepted but lack of a carboxyl anion at C1 in the side chain is not. While the influx carrier for ABA has not been isolated or cloned, the gene for an IAA influx carrier in *Arabidopsis* has been cloned.

2.2.4. Isolation of an IAA Influx Carrier

Attempts to isolate the auxin influx carrier by biochemical means have not been particularly successful. Use of tritiated photoaffinity-labeled IAA (for this methodology, see Appendix 4) led to the identification of two proteins in plasma membrane fractions from a variety of dicots, monocots, and conifers. The proteins (M_r values ~ 40 and 42 kDa, which could be the same protein) occurred in low abundance and were found only in the plasma membrane fractions from those tissues/organs believed to be active in auxin uptake or transport. The proteins have not been characterized further.

Auxin-insensitive mutants, i.e., mutants that do not give the expected response to applied auxin (for isolation of these mutants, see Chapter 22), may be defective in auxin signaling or in auxin uptake or transport. One such mutant in *Arabidopsis*, *aux1* (for auxin resistant 1), shows a reduction in the magnitude of several auxin-specific responses, especially in roots. As shown in Chapter 14, auxin is necessary for the initiation of lateral and adventitious roots (although once initiated, auxin also inhibits root growth). Roots also bend toward gravity (positive gravitropism), a response that is mediated in part *via* auxin (see Chapter 27). The *aux1* mutant shows reduced lateral root initiation and a slower gravitropic response than the wild type, defects that suggest a deficiency in auxin uptake or transport. The wild-type *AUX1* gene has been cloned. It encodes a hydrophobic protein with 10–12 transmembrane domains and a sequence similarity to carrier proteins (permeases) for amino acids in a variety of organisms, including plants. Permeases are active as proton symporters, as is postulated for the auxin influx carrier from biochemical studies (see earlier discussion). Based on these observations, it has been proposed that *AUX1* is the influx carrier for IAA. Data on uptake and efflux of different auxins in *aux1* mutant support this conclusion (see Section 4.5). Moreover, the expression patterns of *AUX1* in roots subjected to gravitropic stimulus confirm that it is involved in auxin transport.

2.2.5. Significance of Carrier-Mediated Uptake of IAA

The uptake of auxin by passive diffusion and/or *via* an influx carrier is an important prerequisite for the polar transport of auxin, which is discussed in Section 4. However, what proportion of uptake is carrier mediated or what role the carrier-mediated uptake plays at the whole plant or organ level is not clear. It is significant that the pH optimum for carrier-mediated uptake (\sim pH 5.0) corresponds with the pH range in the apoplast. It is possible, therefore, that in comparison to diffusion, carrier-mediated uptake is utilized proportionally to a greater extent when auxin concentrations in the apoplast are low or when the pH gradient across the plasma membrane is small. It is also possible that uptake *via* a carrier plays a major role when rapid changes in distribution of a hormone in the plant tissue are needed, as, for example, during tropic responses when IAA must be redistributed laterally in a relatively short time.

3. TRANSLOCATION OF HORMONES

Hormones are transported over short or long distances in the plant, and such movement can be demonstrated by the use of radiolabeled hormones. For example, radiolabeled GA applied to young, fully expanded leaves moves down the stem and up to growing shoot apices (Fig. 13-7). Tritium-labeled brassinosteroids, such as brassinolide and castaestrone, supplied to roots of rice seedlings move up into the stems and are recovered as metabolites. Ethylene alone among plant hormones is believed not to be transported over long distances. However, the immediate precursor of ethylene, ACC, in water-logged rice plants is translocated from the roots to the stem and is converted to ethylene in the aerial parts.

3.1. Translocation by Diffusion

All hormones are translocated over short distances by diffusion. Such transport may occur in the **apoplast**, i.e., is the interconnected system of cell walls and intercellular spaces, or in the **symplast**, i.e., the interconnected system of plasma membrane-bound cells, including the plasmodesmata. Transport velocity is governed by the concentration gradient and by size and polarity of the transported molecule. In the symplast, however, it can be more complex, being also regulated by compartmentation and/or desmotubule in the plasmodesmata. Usually the diffusion velocity in living systems varies between 2 and $5 \text{ mm} \cdot \text{h}^{-1}$.

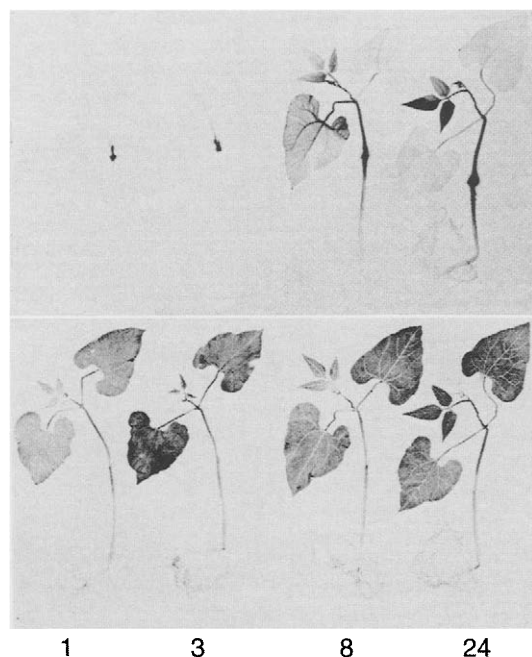


FIGURE 13-7 Movement of GA in bean plants. ^{14}C -labeled GA was applied to the cotyledonary node of the plant, and its distribution with time within the plant was followed by autoradiography. In this technique, plants supplied with radiolabel, after appropriate times, are pressed between sheets of blotting paper and exposed to X-ray film, which when developed shows the location of the radiolabel. (Top) Autoradiograms of plants at different times after supply of radiolabeled GA and (bottom) the same plants photographed in normal light. The plant on the left shows the site of application of radiolabeled GA. From Wareing and Phillips (1981).

An activated diffusion over long distances, involving carrier proteins, is shown by IAA. Such movement is strictly polar and occurs in a basipetal direction in living parenchyma cells. The following sections consider the long-distance translocation of hormones in xylem or phloem first before considering the polar transport of IAA.

3.2. Transport in Xylem or Phloem

Translocation of hormones in xylem or phloem occurs at relatively high velocities (in phloem tissues, for instance, at $20 - 100 \text{ cm} \cdot \text{h}^{-1}$), but is subject to the directionality imposed by the xylem or phloem streams. Translocation in phloem tissue occurs from "source to sink," i.e., from areas of high photoassimilate production, such as fully expanded mature leaves, to areas of high consumption, such as growing root and shoot apices, cambial region, developing inflorescences, and young immature seeds. Translocation in xylem occurs predominantly from roots upward into the shoot.

3.2.1 Demonstration of Xylem or Phloem Transport

Translocation in xylem or phloem is demonstrated by obtaining xylem or phloem sap and analyzing for hormones by immunoassay or GC-MS. Xylem sap is obtained by cutting the stem of a rooted plant about 4–6 cm above the soil level and collecting the exudate from the cut stump (Fig. 13-8A). Phloem sap is usually obtained by making incisions in the bark and collecting the sap via a capillary tube (Fig. 13-8B). Usually, several incisions, one above the other, are required to collect sufficient amounts. Phloem sap collected in this manner is usually contaminated by debris and solutes from cut or injured cells. A method that gives "cleaner" phloem sap utilizes aphids that feed on phloem tissue. The aphids insert their mouth part, the stylet, through the bark into sieve elements (Fig. 13-8E) and feed on the photoassimilate that is being translocated. Feeding aphids release "honeydew" from their rear ends, which can be collected in sufficient quantities for analysis (Fig. 13-8C). A feeding aphid can also be anesthetized by a gentle stream of CO_2 and the stylet cut off by a sharp razor or laser beam. The cut end of the stylet continues to exude sieve tube sap for many hours (Fig. 13-8D).

3.2.2. Analysis of Phloem Exudate

Analysis of phloem exudate or honeydew has revealed the presence of several hormones, as well as their metabolites. For example, phloem exudates from 14 species of eight genera of trees, such as *Acer*, *Carpinus*, *Castanea*, *Quercus*, *Robinia*, *Tilia*, *Sorbus*, and *Alnus*, showed the presence of IAA, ABA, GA, and CK, as well as several of their metabolites by radioimmunoassay. The antibodies used for GA or CK recognized $\text{GA}_{3/7}$ or isopentenyladenine and zeatin, respectively. IAA and ABA, as well as GA_1 , GA_{19} , and GA_{20} were identified by GC-MS in the phloem exudate collected from seedlings of *Pharbitis nil*. Several GAs, both of early 13-hydroxylation and of early nonhydroxylation pathways, were identified by GC-MS in the phloem sap of *Ricinus communis* (castor bean), *Lupinus albus* (white lupin), and *Vigna unguiculata* (cowpea). Zeatin and dihydrozeatin were identified by GC-MS in the phloem sap of white lupin. ABA and its metabolites, phaseic acid (PA) and dihydrophaseic acid (DPA), were reported in the phloem sap from castor bean and white lupin, as well as inflorescence axes of *Yucca* and *Cocos nucifera* (coconut). Jasmonic acid is also known to be translocated over long distances, and it is assumed that it is translocated in the phloem stream, but an unequivocal demonstration of the presence of JA in phloem exudate or honeydew has not been done. Brassinosteroids have been identified in the cambial region of Scots pine (*Pinus sylvestris*), but

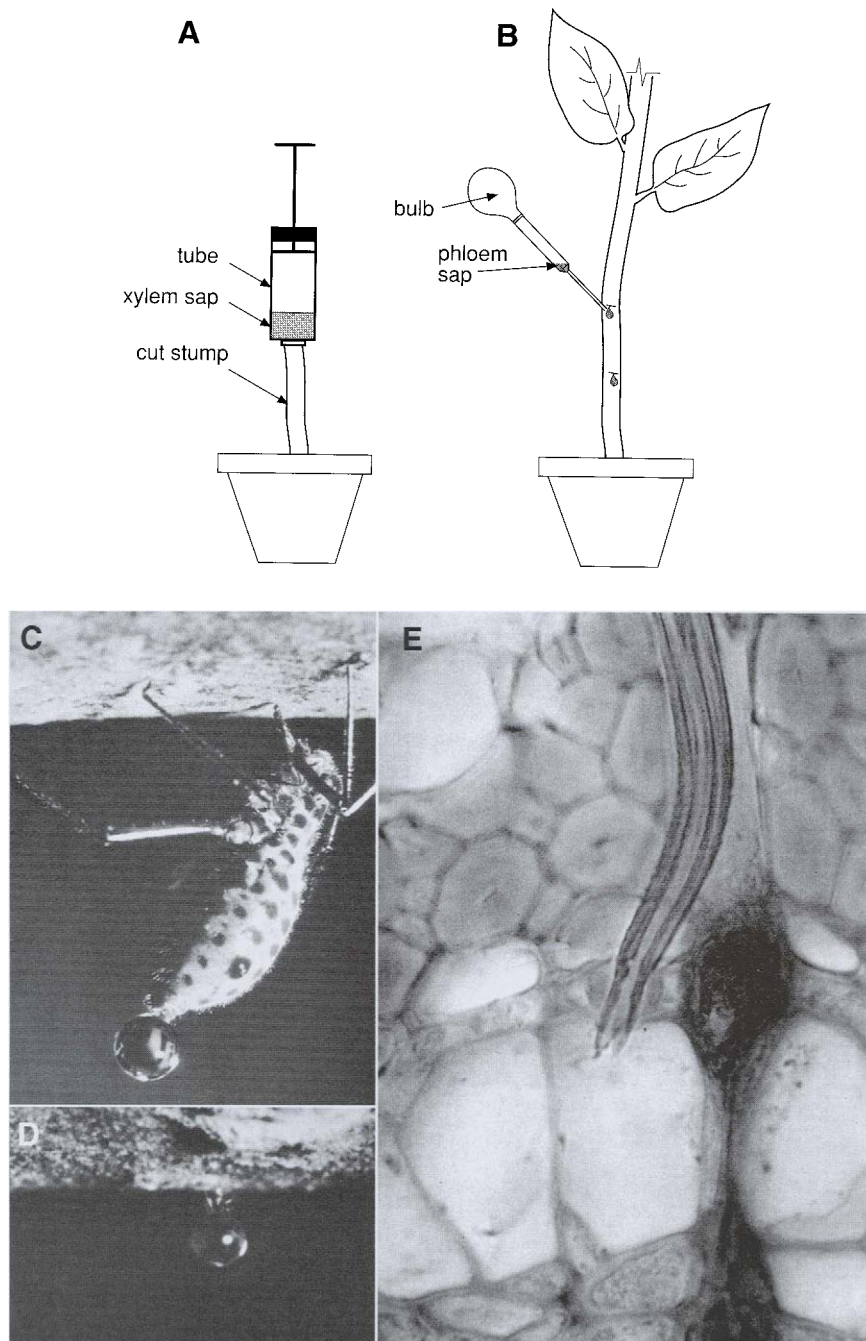


FIGURE 13-8 Collection of xylem and phloem sap. (A) Xylem sap is collected by cutting a stem and collecting the sap that exudes from the cut stump under root pressure. (B) Phloem sap can be collected by making small incisions in the bark with a sharp knife; the exuding sap may be collected by a capillary tube. (C–E) Aphids feed on photoassimilate, which is translocated in the sieve elements. They use the nitrogenous material in the sieve tube sap for their nutrition, but release the sugary solution as honeydew, which can be collected. (C) An aphid is seen feeding on the bark of a tree (part of the branch appears on the top). (D) The body of the aphid has been surgically removed after anesthesia, leaving the cut end of the stylet still in place in the bark and exuding honeydew. (E) The end of the stylet inside the lumen of a sieve element in the inner bark. From Zimmermann and Brown (1971).

it is not clear whether they are transported in the phloem tissue.

3.2.3. Analysis of Xylem Sap

ABA has been reported in the xylem sap of several plants [e.g., castor bean, sunflower (*Helianthus annuus*), maize (*Zea mays*), pine (*Pinus sylvestris*), spruce (*Picea sitchensis*)]. ABA is known to mediate several, although not all, defense responses in plants against water stress (see Chapter 16). Because roots are the first plant organ to detect a water deficit in soil, it has been postulated that a rise in the ABA level in the xylem sap is the signal that is carried from roots upward to aerial parts and triggers protective responses, such as the closure of stomata. Root tips are one of the major sites of cytokinin synthesis in plants. From roots, cytokinins are translocated upward to stem and leaves. Thus, there are many reports of their presence in xylem sap. The major cytokinins identified in xylem sap, both by immunoassays and GC-MS, are zeatin riboside and isopentenyladenine. In contrast to ABA and cytokinins, there is little information on the transport of IAA, GAs, jasmonic acid, or brassinosteroids in the xylem stream, and it is not clear whether such transport occurs. However, gibberellins and brassinosteroids, supplied via roots, are picked up and apparently translocated to aerial parts.

3.2.4. Hormone Concentrations in Xylem or Phloem Sap

Studies on the translocation of hormones in xylem or phloem are relatively few, and there is very little information on the total amounts or proportions of free hormones vs metabolites that are translocated. In one study, free IAA levels in the phloem exudate were reported to vary between 40 and 520 nmol · liter⁻¹; it was also reported that the concentrations of free and conjugated IAA varied with the developmental state of the plant. The concentrations of GA₁, GA₁₉, and GA₂₀ in the phloem exudate of *Pharbitis nil* seedlings were reported to increase in seedlings that had been induced to flower. ABA concentrations in xylem sap of well-watered maize and sunflower plants are about 10 nM; amounts that can rise by one to two orders of magnitude by mild drying of the soil. CK concentrations in xylem sap also vary with the physiological or developmental state of the plant. For example, decapitation of the shoot apex causes a surge in CK concentration in the xylem stream. An increase in CK concentration is observed with the initiation of flowering, followed by a decrease at the onset of whole plant senescence.

4. POLAR TRANSPORT OF AUXIN

IAA alone among all plant hormones is also known to move long distances in the plant in a strictly polar, basipetal direction (for the first demonstration of such movement, see Fig. 5.1, Chapter 5). Treatment with chemicals that disrupt such polar transport of IAA or mutants that are defective in such transport show clearly that the polar movement of IAA has important consequences in plant development and morphogenesis and in certain responses to environmental factors. For example, establishment of polarity and bilateral symmetry in embryo development, vascular tissue differentiation, apical dominance, induction of lateral and adventitious roots, and tropic responses to directional light and gravity are directly traceable to the polar movement of IAA in the plant. Some of these phenomenon are considered in the next chapter and in Section V. Here we will continue with the demonstration, characteristics, and mechanism of polar transport of auxins.

4.1. Demonstration of Polar Transport of Indoleacetic Acid

The polar transport of IAA can be demonstrated using hypocotyl/epicotyl or coleoptile segments and placing them between a donor and a receiver agar block (Fig. 13-9). Radiolabeled IAA applied to the donor block moves in a basipetal direction. If the segment is placed horizontally, IAA still moves basipetally; however, if the segment is inverted, there is hardly any movement of the label. The amount of radiolabel accumulated in the receiver block can be plotted as a function of time and can be used to calculate the velocity and flux (or rate) of transport.

Analysis of radiolabeled species in the receiver blocks shows that free IAA is being translocated and not a conjugated or an otherwise modified form. Also, although cut segments are an easier material for *in vitro* experiments, use of intact pea or maize plants has shown that IAA moves similarly in whole plants.

4.2. Directionality of Transport in Shoots and Roots

The polar transport of IAA in shoots occurs predominantly in a basipetal direction, i.e., from the shoot tip toward the root. In roots, also, the movement is basipetal, i.e., from the root tip toward the root-shoot junction (the base of the root). The specific files of cells involved in such basipetal transport are not clear. Experiments

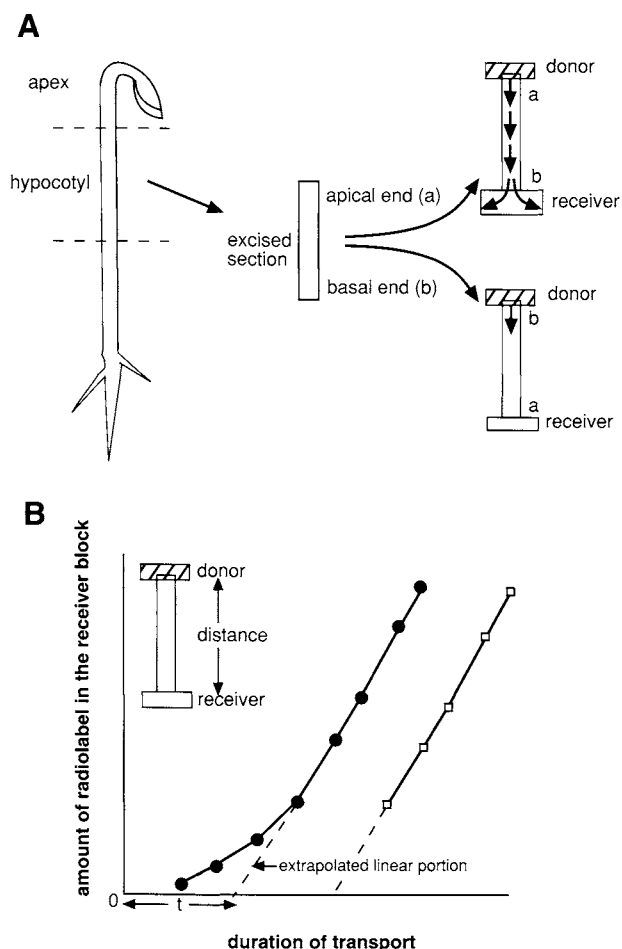


FIGURE 13-9 Polar transport of IAA using hypocotyl segments from squash (*Cucurbita pepo*). (A) A hypocotyl segment is placed between a donor block containing radiolabeled IAA and a receiver block. If the segment is kept right side up, there is movement (shown by arrows) of radiolabel into the receiver block. If the segment is inverted, there is little or no movement of the radiolabel. (B) Velocity and flux of transport. Hypocotyl segments are placed right side up, and receiver blocks are sampled at different times following the start of experiment. Radioactivity in the receiver blocks can be plotted against the duration of transport. The velocity of transport (distance/time) can be calculated by extending the slope of accumulated radioactivity to the abscissa, which gives the time (t) at which the steady-state transport of radioactivity started. The amount of radiolabel translocated per unit time, after the steady state has been reached, gives the rate or flux of transport. The second plot (on the right) was obtained with a stem segment that was twice as long as the one on the left. The slope of the accumulated auxin, or the rate of transport, in the two cases is the same, indicating that the velocity and rate of transport are independent of stem length. Modified from Lomax *et al.* (1995) and Thimann (1977) with kind permission from Kluwer.

using tritiated- or tritiated- and photoaffinity-labeled IAA and microautoradiography have been performed only with a few plants. Available data suggest that in shoots, parenchyma cells close to or associated with the vascular cylinder (starch or bundle sheath cells)

are the sites of translocation, whereas in roots, cortical and epidermal cells seem to be the primary sites for basipetal translocation (Fig. 13-10).

As mentioned earlier in Section 2, IAA and its metabolites are also translocated in the phloem tissues. Thus, while in the shoot, both the polar stream and the vascular stream are basipetally directed; in roots the situation is more complex. In the so-called "fountain" model of IAA transport in roots, the vascular stream is directed acropetally toward the root tip, whereas the polar stream is directed basipetally toward the root-shoot junction (Fig. 13-10). Moreover, the vascular stream may also have a polar (or activated diffusion) component closer to the root tip, as sieve elements are replaced by procambial or parenchyma cells of the central cylinder. It is postulated that the acropetally

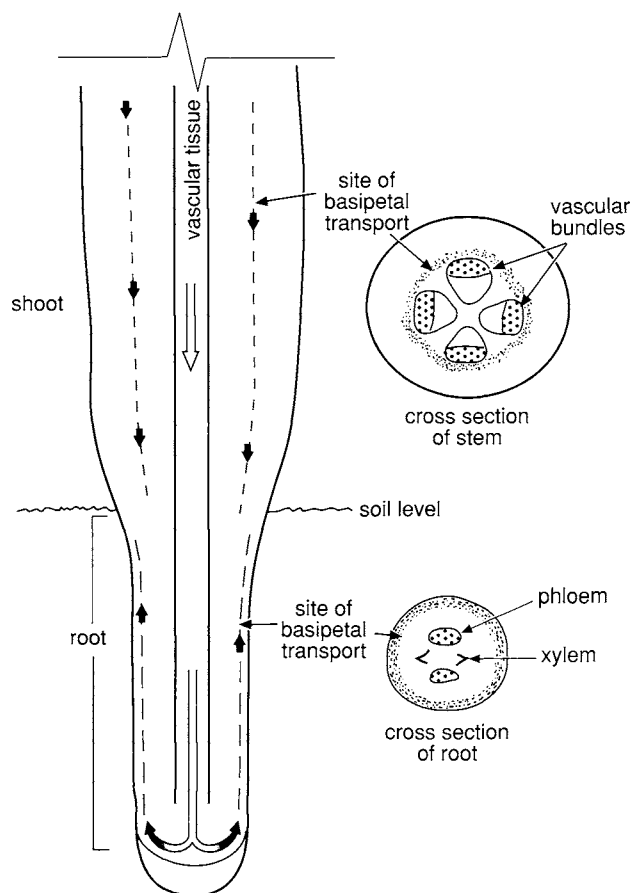


FIGURE 13-10 Transport of IAA in a dicot plant. In the shoot, the polar, basipetal transport of IAA is directed toward the root and occurs in parenchyma cells associated with the vascular cylinder (solid arrows). In roots, a similar polar transport of IAA occurs in peripheral tissues of the cortex and epidermis from the root tip toward the root-shoot junction. In both shoot and root, IAA and its metabolites are also translocated in the phloem or procambial tissue (large open arrows) toward the root tip.

translocated IAA arriving at the root tip is redirected back upward, or basipetally, in the cortex and epidermal cells. What happens at the root-shoot junction where the two polar streams meet is not known. Also, it is not clear whether IAA is exchanged between the stream moving in the vascular tissue and the polar stream in parenchyma cells.

4.3. Characteristics of Polar Transport

The polar transport of auxins has several characteristics.

i. It requires energy in the form of ATP. Anaerobic conditions or metabolic poisons, such as cyanide and 2,4-dinitrophenol (DNP), which inhibit oxidative phosphorylation in mitochondria, inhibit the polar transport. The requirement of energy is chiefly for priming the auxin uptake *via* the influx carrier, which is driven by a proton electrochemical gradient between the apoplast and the symplast.

ii. Transport occurs in living parenchyma cells. The velocity of transport, measured in experiments as in Fig. 13-9B, varies with tissue and environmental conditions from 5 to 20 mm · h⁻¹, which is about three to four times greater than simple diffusion. Similar velocities have been recorded in intact plants.

iii. If stem segments of different lengths are used in transport assays *in vitro*, it can be shown that not only does the velocity of transport remain the same [although the initial lag time (*t*) increases in proportion with the length of the segment], but the rate or flux of transport, i.e., the total number of molecules transported per unit time, also remains the same. This is shown in Fig. 13-9B, which shows basipetal transport through two segments, one twice as long as the other. If the movement was by simple diffusion, the longer segment would mean slower diffusion and a lesser slope of the accumulated auxin.

iv. What is most striking is that accumulation of IAA can occur in the receiver block against a concentration gradient, supporting the idea that auxin transport through tissues is not by simple diffusion, but by a facilitated diffusion that involves energy. If the auxin concentration in the receiver block is raised to the same level as in the donor block, such that there is no chemical gradient, the movement of auxin and accumulation in the receiver block still continue.

4.4. Polar Transport is Mediated *via* Specific Efflux Carriers

The data just described are explained by a hypothesis that postulates that the basipetal transport of IAA is mediated *via* specific and saturable carrier proteins.

These proteins are localized primarily at the morphologically lower end of the participating parenchyma cells in the shoot tissues. IAA enters the cells in a protonated form by diffusion and/or as an anion *via* auxin influx carriers, and such entry occurs from all sides of the cell. Inside the cell, the IAAH dissociates into IAA⁻ and H⁺ according to the prevailing cytosolic pH. The plasma membrane is negatively charged and inhibits IAA⁻ from leaving the cell, except at specific sites, where efflux carriers are located. These efflux carriers are located on the morphologically basal end of the cell and are unidirectional in their export (Fig. 13-11). It follows that if the efflux carriers are inhibited in their action, IAA⁻ accumulates in the cell. This hypothesis, proposed independently by Rubery and Sheldrake (1974) and Raven (1975) in the United Kingdom, referred to as the chemiosmotic theory of polar auxin transport after a similarly named theory for solute transport, has been vindicated through the last quarter of century and recently achieved its crowning glory, the cloning of genes purporting to encode the auxin efflux carrier.

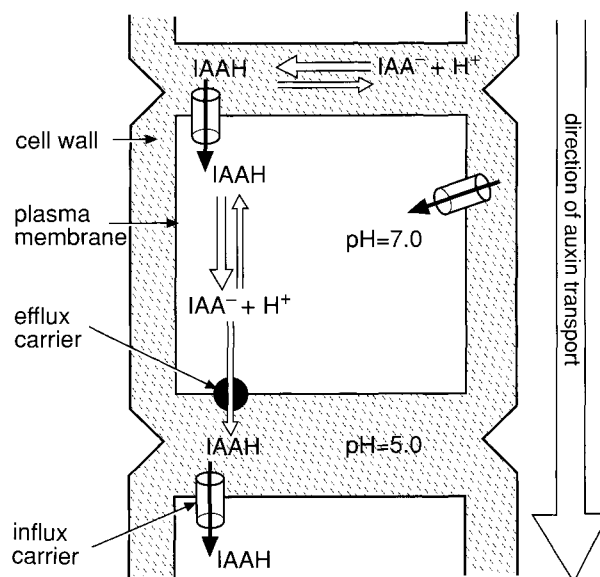


FIGURE 13-11 A model for polar auxin (IAA) transport through a file of parenchyma cells. IAA enters the cells from all sides in its protonated form and/or *via* an auxin influx carrier (e.g., AUX1 or related proteins). On exposure to cytoplasmic pH, auxin undergoes deprotonation and is effectively trapped inside the cell. Auxin can leave the cell only through the activity of an auxin efflux carrier located specifically on the basal plasma membrane. Note that this is not strictly a symplastic transport of IAA—transport occurs from cell to cell with intermediation of the apoplast. Apoplastic and cytosolic pH are indicated, and the relative size of the arrows indicates the predominant form in which IAA occurs in the apoplast or in the cytosol. From Lomax *et al.* (1995), as modified by Leyser (1999), and with kind permission from Kluwer.

4.5. Polar Transport of Different Auxins Is Differentially Regulated by Auxin Influx and Efflux Carriers

Among the naturally occurring auxins, only IAA and indolebutyric acid (IBA) are transported in a polar manner. Phenylacetic acid (PA) does not show polar transport; indeed, it acts as an inhibitor of the polar transport of IAA. Polar transport is also shown by biologically active synthetic auxins, such as 1-NAA, but very little by 2,4-D. The biologically inactive analogs, such as 2-NAA, or 2,6-dichlorophenoxyacetic acid, or benzoic acid, are not transported.

Uptake and efflux studies using tobacco cells in culture have indicated some striking differences in the uptake and efflux of synthetic auxins. While 2,4-D, like IAA, is taken up by the influx carrier, it is not secreted by the efflux carrier; hence 2,4-D accumulates in cells. This may be the reason for its relatively poor capacity for polar translocation. In contrast, 1-NAA, being highly lipophilic, enters the cell mainly by diffusion, not *via* the influx carrier; moreover, it is secreted, like IAA, by the efflux carrier. These differences in the uptake and efflux properties of auxins can be utilized for study of the role of polar transport in specific auxin-mediated responses. For instance, the *aux1* mutant of *Arabidopsis* is defective in root gravitropism because it is unable to take up or transport IAA effectively. While *aux1* mutants cannot be restored to normal gravitropism by treatment with exogenous IAA or 2,4-D, they can be so restored by application of 1-NAA (Fig. 13-12).

To summarize, the polar transport of IAA is accomplished by a combination of uptake and efflux. IAA is taken up by cells in its protonated form by passive diffusion and in the anionic form by the activity of influx carriers, which are specific to auxins and are driven by the proton gradient that exists between the apoplast and the symplast. In the cytoplasm, IAA occurs preeminently in the anionic form, which can leave the cell only through specific efflux carriers located on the basal plasma membrane. In contrast to influx carriers, efflux carriers do not require an input of energy—diffusion through them is concentration dependent.

4.6. Polar Transport Is Inhibited by Chemicals

Several synthetic substances inhibit the polar transport of IAA. Applied along with IAA or in the path taken by IAA, they inhibit the transport at the site of their application. A subset of these compounds, known as phytotropins, have the common structural

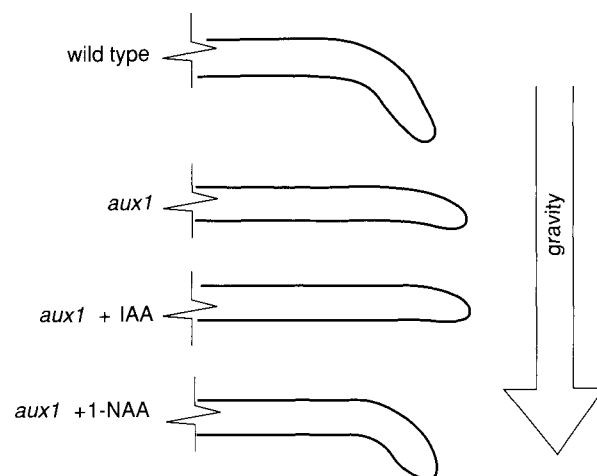


FIGURE 13-12 A schematic representation of the gravitropic response in *Arabidopsis*. The roots of wild-type plants, placed horizontally, respond to gravity by growing downward. Those of the *aux1* mutant are impaired in this response, but are restored to normal response by treatment with 1-NAA, but not by IAA. Drawn from data in Marchant *et al.* (1999).

theme of benzoic acid ortho-linked to a second aromatic ring system (Fig. 13-13). *N*-1-Naphthylphthalamic acid (NPA) is the most widely used phytotropin, although 1-pyrenoylbenzoic acid (PBA) is the most active compound. Application of phytotropins also causes the severe disruption of plant development (e.g., stunting, inhibition of root growth), as well as its ability to respond to gravi- and phototropic signals. Other polar transport inhibitors, 2,3,5-triiodobenzoic acid (TIBA), and the morphactins (e.g., 2-chloro-9-hydroxyfluorene-9-carboxylic acid) deviate structurally from the definition and are not considered phytotropins. TIBA also does not interfere with gravitropic and phototropic responses.

Inhibition of polar transport in stems or coleoptiles by phytotropins leads to an accumulation of IAA above the site of application (Fig. 13-14). In the roots, such application blocks the movement of IAA basipetal to the site of application. If applied closer to the root tip, it may also block acropetal transport in procambial cells in the central cylinder. Blockage of auxin efflux and an accumulation of IAA[−] by NPA (or TIBA) can be shown in membrane vesicles as well. Such accumulation clearly indicates that the efflux carrier, not the influx carrier, is being inhibited.

4.6.1. Auxin Efflux Carrier May Be Part of a Multiprotein Complex

Not much is known about how auxin transport inhibitors act. Nonphytotropins, such as TIBA, which

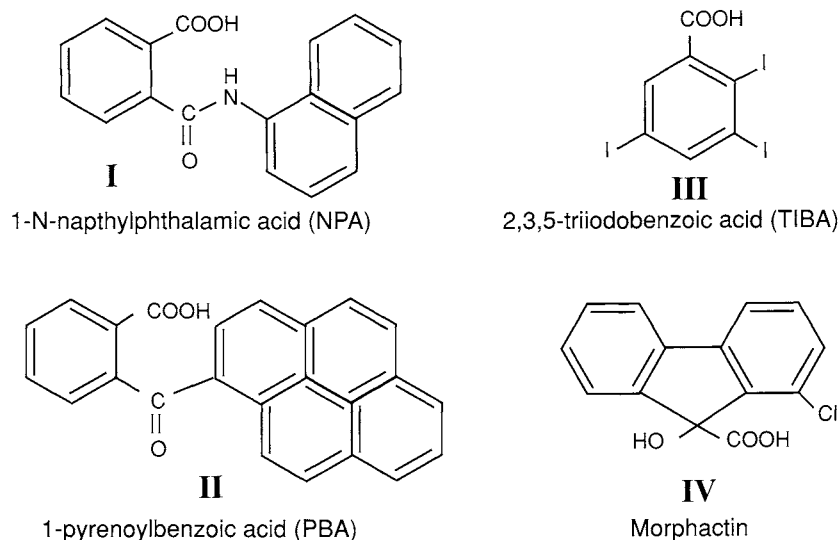


FIGURE 13-13 Chemical structures of some inhibitors of polar transport of IAA.

are weak auxins and show some polar transport, may bind to the efflux carrier itself. In contrast, phytotropins, are thought to bind to a regulatory protein, which is associated with, but is separate from, the efflux carrier and modulates its activity (Fig. 13-15). Two types of evidence support the idea that the NPA-binding protein and the efflux carrier are separate proteins.

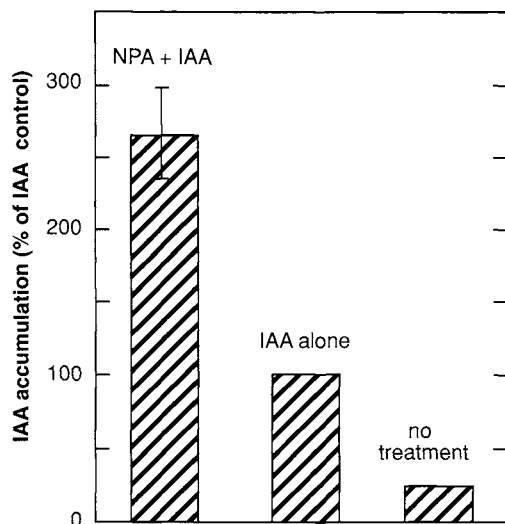


FIGURE 13-14 Accumulation of IAA in cut coleoptile segments treated with naphthylthalamic acid (NPA). Coleoptile segments were treated with NPA (5 μ M) plus IAA (1 μ M), IAA alone (IAA control), or no treatment (endogenous control). Treatment was terminated after 35 min, and IAA accumulated in the tissue was extracted, measured, and plotted. Values are expressed as the percentage of IAA control set at 100% and are means of four pairs of simultaneous experiments; error bar indicates \pm SE. With permission from Clausen *et al.* (1996), ©1996 Munksgaard International Publishers Ltd., Copenhagen, Denmark.

(i) IAA efflux and polar transport are stopped if hypocotyl segments are treated with inhibitors of protein synthesis (e.g., cycloheximide) or inhibitors of Golgi vesicle secretion (e.g., monensin or brefeldin A), but the number of NPA-binding sites (see Section 4.6.2) remains unaffected. (ii) Treatments of membrane fractions with KI and Na_2CO_3 remove most of the NPA-binding activity from microsomal preparations, which indicates that the NPA-binding protein is loosely bound to the membrane but is not integral to it.

More complicated models are possible, and the concept has grown that the auxin efflux carrier is part of a multiprotein complex.

Hypocotyl segments that have been preloaded with ^{14}C IAA, and subsequently treated with cycloheximide or monensin (or brefeldin A), show an inhibition in the rate of auxin efflux relative to untreated controls. Such inhibition suggests that the efflux carrier protein (or some essential component associated with it) has a rapid turnover, or a short half-life, and needs to be continually recycled to maintain the efflux at a steady rate. In contrast, the influx carrier, is more stable and has a long half-life. These data need independent confirmation, but, if true, have important consequences in the redirection of polar transport in plants.

4.6.2. NPA-Binding Proteins and Their Natural Analogs

The regulation of auxin efflux carriers is of importance in plant growth and development because it allows localized changes in the concentration of IAA, e.g., during initiation of lateral roots or for lateral redistribution of auxin as in a tropic response. Hence, much

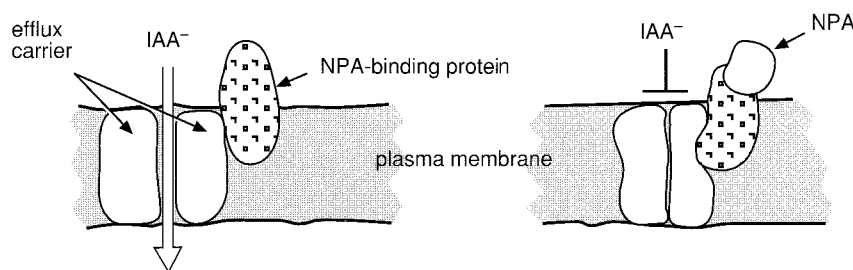


FIGURE 13-15 A simple model showing the auxin efflux carrier and the NPA-binding protein. The efflux carrier is an integral membrane protein, whereas the NPA-binding protein is loosely anchored to the membrane (left). On binding of NPA, the NPA-binding protein undergoes a conformational change which, in turn, modulates the conformation of the efflux carrier protein such that the transport of IAA is inhibited (right).

effort has gone into isolation and characterization of phytotropin-binding proteins and, because phytotropins are synthetic compounds, isolation and characterization of their natural analogs. However, despite much effort, these attempts have not been very successful.

Binding assays, similar to those used for hormone binding to its receptor, show that radiolabeled NPA binds specifically and with high affinity to plasma membrane fractions from parenchyma cells of several plants. Scatchard plots indicate a single binding site, which means that binding is occurring to a single class of proteins (for hormone-binding assays and Scatchard plots, see Appendix 4). However, the protein(s) has not been purified.

The search for natural regulators of auxin efflux carriers, i.e., compounds that inhibit auxin efflux and also compete for a NPA-binding site, has turned up some phenolic substances, especially a flavonoid, quercetin. When treated with quercetin, pea stem

segments accumulate radiolabeled IAA, about 40% more than the control. However, quercetin is required in a large concentration (about 10 μ M) and it is doubtful that it is the natural regulator of polar IAA transport. Subsequent studies have shown that sulfated forms of quercetin are effective at lower concentrations.

To gain further insight into NPA-binding proteins, mutagenized *Arabidopsis* plants were screened for tolerance to relatively high doses of NPA. Several *tir* (for transport inhibitor response) mutants, belonging to different loci, were isolated. One of these mutants, *tir3* (see Table 13-1), shows a much reduced polar transport of auxin, only about 30% of that in the wild-type plant. Membrane fractions isolated from the mutant also show a reduced number of NPA-binding sites—about threefold lower than in the wild type. Interestingly, *tir3* mutants show a drastic curtailment in lateral root production, which indicates that the TIR3 protein has a role in auxin transport. The TIR3

TABLE 13-1 IAA Polar Transport Mutants in *Arabidopsis*

IAA transport mutants	Possible site of lesion	Ref.
<i>aux1</i>	Auxin influx carrier, active in roots	Bennett <i>et al.</i> (1996).
<i>tir3</i>	Possible defect in NPA-binding protein	Ruegger <i>et al.</i> (1997).
<i>pin1</i>	Auxin efflux carrier, specific to shoots	Okada <i>et al.</i> (1991)
<i>monopteros (mp)</i>	Defective embryo development and defective vasculature in adult plant	Hardtke and Berleth (1998)
<i>lop1</i>	Defective leaf vasculature and morphology	Carland and McHale (1996)
<i>eir1/pin2</i>	Auxin efflux carrier, specific to roots	Müller <i>et al.</i> (1998), Luschnig <i>et al.</i> (1998), Utsuno <i>et al.</i> (1998), Chen <i>et al.</i> (1998)

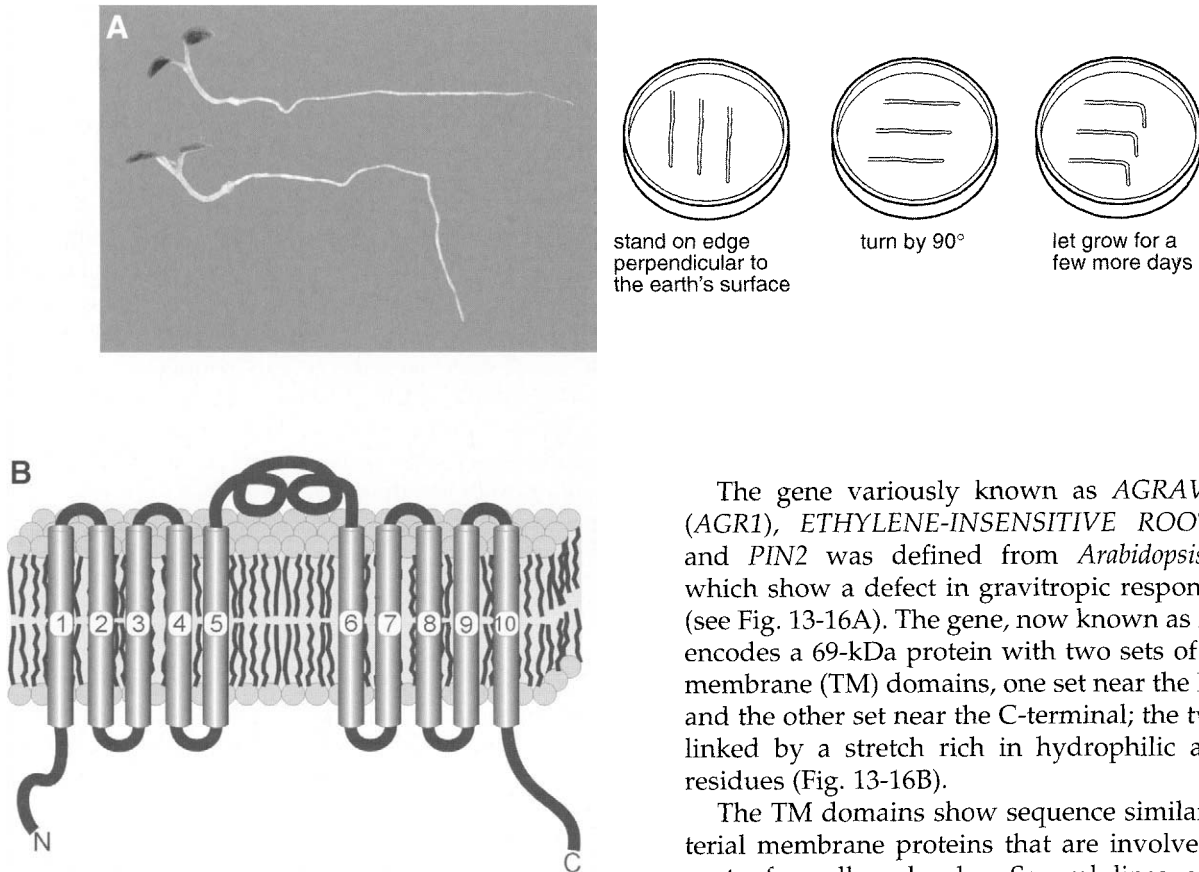


FIGURE 13-16 (A) Wild-type *Arabidopsis* (bottom) and the mutant *eir1-3* (top). For these photographs, seedlings were grown on agar in petri plates placed vertically on edge (perpendicular to the earth's surface) for 5 days. Subsequently, the petri plates were reoriented so that the roots were parallel to the surface of the earth. The photographs show direction of new root growth 48 h after reorientation. The protocol is shown in the accompanying sketch. In the wild type, root and shoot growth are reoriented in response to gravity; in the *eir1-3* mutant, while the shoot shows a normal gravi response, the roots do not respond to gravi stimulation. Photograph from Luschnig *et al.* (1998). (B) Schematic structure of EIR1/PIN2 protein in *Arabidopsis*. From Müller *et al.* (1998).

gene has not been cloned, and the function of the TIR3 protein is unknown, but it is thought that it might be one of the components of the efflux-carrier complex.

4.7. Cloning of the Gene for an Auxin Efflux Carrier

While the isolation of phytochrome-binding proteins or the natural regulators of the auxin efflux carrier by biochemical means has proven difficult, in 1998, in a remarkable coincidence, four separate groups cloned a gene that encodes an auxin efflux carrier. Table 13-1 lists several mutants in which polar IAA transport is disrupted.

The gene variously known as *AGRAVITROPIC1* (*AGR1*), *ETHYLENE-INSENSITIVE ROOT1* (*EIR1*), and *PIN2* was defined from *Arabidopsis* mutants, which show a defect in gravitropic response in roots (see Fig. 13-16A). The gene, now known as *EIR1/PIN2*, encodes a 69-kDa protein with two sets of five transmembrane (TM) domains, one set near the N-terminal and the other set near the C-terminal; the two sets are linked by a stretch rich in hydrophilic amino acid residues (Fig. 13-16B).

The TM domains show sequence similarity to bacterial membrane proteins that are involved in transport of small molecules. Several lines of evidence suggest that *EIR1/PIN2* is involved in the transport of IAA anion.

i. The expression of *EIR1/PIN2* mRNA is restricted to root tissues, specifically to root tip and elongation zone, areas known to be involved with the gravitropic response.

ii. The pattern of protein expression in root cells is consistent with the path predicted for the basipetal transport of auxin in the root. The protein is localized exclusively to the plasma membrane of the root tip cortical and epidermal cell files, starting three to five cells back from the center of the root apical meristem. Moreover, in cortical cells, the protein is localized at the membrane in contact with periclinal walls between cortical and cortical and epidermal cells and in both cell types in the membrane associated with the anticlinal walls. In contrast, the protein shows a diffuse occurrence in the mutant roots (Fig. 13-17).

iii. Another mutant of *Arabidopsis*, *pin1* (see Table 13-1), is characterized by inflorescences that terminate in pin-like structures with little or no initiation of floral buds. This defect is associated with reduced polar auxin transport in inflorescence, suggesting that the *PIN1* protein is required for polar auxin transport. The *PIN1* gene is a member of the same family as *EIR1/PIN2*, except that it is expressed primarily in shoot

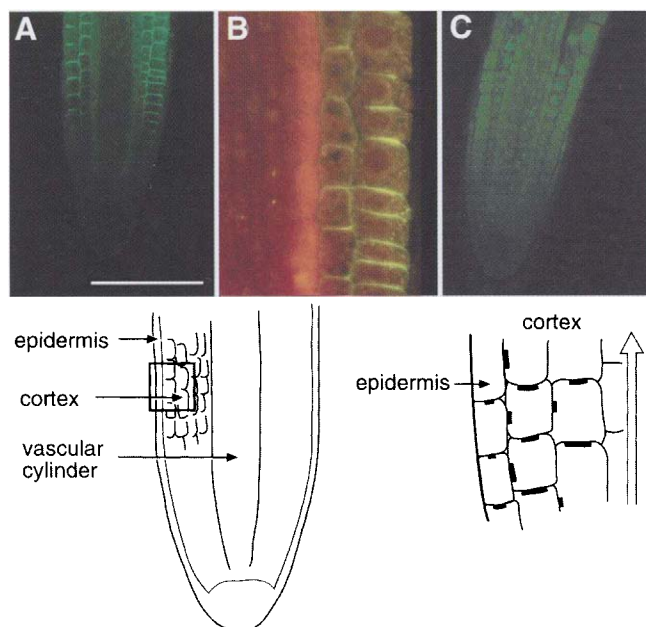


FIGURE 13-17 Localization of EIR1/PIN2 protein in *Arabidopsis* root. (Top) Immunostaining of wild-type (A and B) and mutant (C) *Arabidopsis* root tips with fluorescent antibodies prepared against part of the conserved transmembrane domains of EIR1/PIN2 protein, imaged by confocal laser-scanning microscopy. (A and B) The EIR1/PIN2 protein is localized in the elongation zone of the root, and in that zone at the periclinal walls of cortical cells and at the anticlinal walls of cortical and epidermal cells. In (B) some tissue autofluorescence is seen as well. (C) In the mutant root, the protein shows only weak and diffuse fluorescence. Bar in (A): 100 μm . From Müller *et al.* (1998). (Bottom) A schematic representation of the distribution of EIR1/PIN2 protein in the wild-type root. Note that the fluorescent signal (black bars) is shown along the anticlinal walls of cortical and epidermal cells and periclinal walls between cortical and epidermal cells. Open arrow, direction of auxin transport.

tissues, whereas *EIR1/PIN2* is expressed in roots. An immunocytochemical localization of PIN1 protein, using fluorescent antibodies, in inflorescence axes of *Arabidopsis* shows a similar cellular polarity as shown for EIR1/PIN2 protein in root cortical and epidermal cells. Still another gene, *PIN3*, has been cloned, and many other homologous clones have been identified in *Arabidopsis*. Thus, it appears that auxin efflux carriers are encoded by a family of genes, members of which are expressed in different tissues and/or at different developmental stages.

The cloning of genes for an auxin influx carrier and an efflux carrier has opened the way for a functional characterization of their encoded proteins and studies into regulation of their expression. Both the AUX1 protein and EIR1/PIN2 are integral membrane proteins, but there is little sequence similarity between the two. *AUX1* is the only gene cloned for an auxin influx carrier so far, and it is expressed predominantly

in root tissues; thus, it is likely that related genes exist that are expressed in other tissues/organs.

4.8. Efflux Carriers Can Be Randomized and Rearranged

The location of auxin efflux carriers at the basal ends of cells is not immutable. Under certain conditions, polar IAA movement may be lost or acquired. The loss may mean randomization of the efflux-carrier proteins, and gain may mean increased localization on the basal ends of cells. In this connection, the rapid cycling of the efflux carrier protein, or an essential component of the efflux-carrier complex, is especially significant. We will consider this phenomenon, as well as the possibility that IAA sets up its own channels, a phenomenon known as canalization, in Chapter 14.

5. CHAPTER SUMMARY

Uptake of undissociated hormones by passive diffusion occurs freely across cell membrane, subject only to concentration gradient and the molecular characteristics of the diffusing species. Auxins and ABA are also taken up *via* their respective carrier proteins. The motive force for such uptake, at least for auxins, is provided by the proton gradient between apoplast and symplast established by the activity of proton ATPases. Experiments with isolated plasma membrane vesicles suggest that the auxin carrier functions as a symport for IAA^- and 2H^+ . Hormones and some of their metabolites are translocated long distance in the plant in vascular tissues at high velocities and in the direction in which xylem or phloem streams are translocated. Among the natural hormones, only IAA and IBA also show a highly directed polar transport, which is strictly basipetal both in shoots and roots and occurs in parenchyma cells at rates three to four times higher than those for diffusion. The polar transport of IAA has enormous implications for the normal growth and development of plants and responses to gravi- and phototropic stimuli. The polar movement of auxins involves uptake *via* passive diffusion and/or an influx carrier and secretion of the anion *via* efflux carriers that are localized on the basal end of transport-competent cells. Such localization of efflux carriers is not immutable, however, and can change in response to developmental or environmental cues. Several synthetic substances, such as the phytochrome NPA, block the action of efflux carriers with the result that auxin accumulates in the tissue acropetal to the site of application. Such accumulation by natural inhibitors of polar transport may be important in raising local

levels of auxins or in their redirection. Attempts to isolate NPA-binding proteins and/or the natural regulators of efflux carriers by biochemical means have not been very successful, but a genetic approach has led to the cloning of genes for both a putative auxin influx carrier and an auxin efflux carrier. Several genes encoding the efflux carrier have been isolated already and more are likely to follow; it is also likely that the influx carrier is encoded by multiple genes. Cloning of these genes is an important breakthrough, which is likely to lead to a clearer understanding of the polar transport of auxins, as well as the natural factors that regulate it.

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1. INTRODUCTION

Auxins play important roles in many developmental processes where axiality (i.e., setting up of an axis, such as apical-basal axis) or polarity (an axis where the two ends are morphologically distinguishable, e.g., root-shoot axis) is involved. These processes involve a sense of directionality, and plants use the polar transport of indoleacetic acid (IAA) in stems and roots to find direction. Many developmental processes not only involve auxins, but also an interaction of auxins with other hormones, especially cytokinins (CKs). An interaction between two (or more) hormones, sometimes referred to as "cross communication," underlies the orderly development of many tissues and organs in plants. The polar transport of IAA is covered in Chapter 13. This chapter discusses several topics where axiality or polarity and interaction of auxins with cytokinins are involved. These topics are covered in individual sections, but before considering them, some aspects of auxin and cytokinin homeostasis are reviewed first.

SECTION I. AUXIN AND CYTOKININ HOMEOSTASIS

The orderly growth of a plant requires that hormonal levels be precisely regulated in different tissues/organs during development. Such regulation is part of hormonal homeostasis, and deviations from "normal" levels result in severe developmental abnormalities. This is especially well illustrated for hormones such as IAA and cytokinins.

1. INDOLEACETIC ACID (IAA) OR CYTOKININ OVERPRODUCERS

Although synthesis mutants for IAA *per se* and cytokinins are not known, there are many mutants in which the regulatory control of free IAA levels by conjugation and/or irreversible breakdown is insufficient to maintain IAA homeostasis (see Section 5.3, Table 6-2 in Chapter 6). Similarly, there is at least one *Arabidopsis* mutant, *amp1*, where cytokinin homeostasis is lost (see Section 7, Chapter 8). These mutants show much higher levels of IAA and CKs, respectively, than their wild-type counterparts. Transgenic plants expressing bacterial genes for the synthesis of IAA (*iaaM iaaH* genes) or cytokinin (*ipt* gene) (see Appendix 2) often show relatively mild phenotypes, probably because of compensatory control, but when these genes are overexpressed, they may cause loss of IAA or CK homeostasis and provide data comparable to those from overproducing mutants.

Auxin overproduction in either mutants or transgenic plants results in abundant root initiation, but also a curtailment in root elongation. In shoots, it leads to a stunted habit, suppression of lateral bud growth, epinasty of leaves, and possible sterility due to lack of flower development.

Plants overproducing cytokinins also show multiple defects, the most consistent being enhanced lateral branching, retarded root development, and delayed senescence of leaves. If the amounts of cytokinins produced are overly excessive, shoot elongation may be restricted, there may be a severe loss of apical dominance, leaves may be small and rounded, and plants may not be able to form roots. Other effects include expression of genes for defense-related proteins (e.g., chitinase).

2. INCREASED IAA LEVELS REDUCE FREE CYTOKININ LEVELS AND VICE VERSA

Tissues/plants transformed with *iaaM iaaH* genes and overproducing IAA show lower levels of endoge-

neous cytokinins than untransformed controls. Similar data are obtained by exogenous application of auxins, such as 1-NAA or 2,4-D, to calli or leaf explants. A reduction in free cytokinin may be due to an inhibition of biosynthesis, enhanced inactivation by conjugation/breakdown, or both. Most data indicate that in plants overexpressing *iaaM iaaH* genes, the metabolic profile of inactivation, i.e., the relative proportion of conjugated cytokinins vs cytokinin breakdown products (by the activity of cytokinin oxidase), remains unaffected. There is, however, a reduced synthesis of cytokinins and, overall, a reduced content of cytokinins and cytokinin metabolites in transformed than in untransformed plants (Fig. 14-1). Similarly, in whole plants or tissues transformed with *ipt* gene and overproducing cytokinins, the levels of free IAA and, in some cases, conjugates are reduced. The enzymes for IAA biosynthesis in higher plants, except for nitrilases are unknown (see Chapter 6); also unknown are the enzymes for cytokinin biosynthesis (but see Chapter 8). Hence, it is not possible to provide direct proof for these observations.

SECTION II. AUXINS AND EMBRYO DEVELOPMENT

Auxins play two separate and distinct roles in embryo development: (i) setting up the root-shoot axis and bilateral symmetry and (ii) induction of cell divisions in tissue explants or cell cultures during somatic embryogenesis. It is only the first role in which polar transport of IAA is involved.

1. APICAL-BASAL POLARITY AND BILATERAL SYMMETRY

As mentioned in Chapters 1 and 3, dicot embryos typically go through several stages described as globular, heart-shaped, torpedo, and so on during their development. A root-shoot axis develops between the globular and the heart stage by oriented divisions and elongation of cells between the shoot pole, consisting of the putative shoot apex and cotyledons, and the

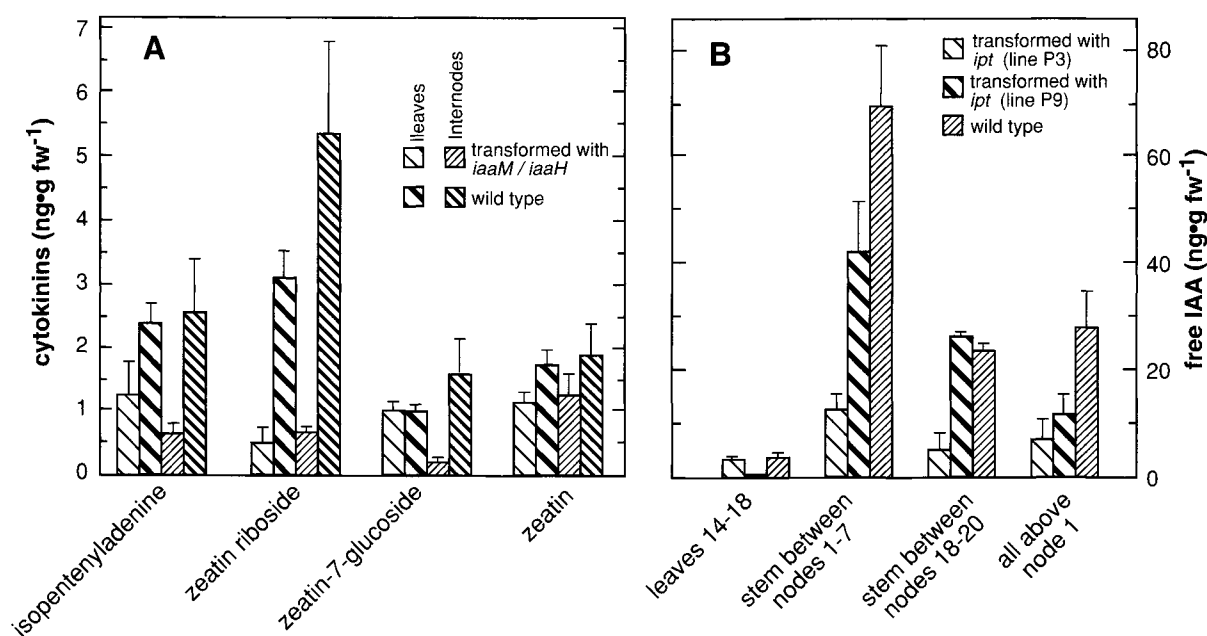


FIGURE 14-1 Cytokinin or IAA concentrations in tobacco plants transformed with *iaaM iaaH* genes or the *ipt* gene under the control of a constitutive 35S promoter. (A) Cytokinin concentrations in young leaves (1–7) and internodes (between nodes 1–11) of wild-type and tobacco plants transformed with *iaaM iaaH* genes. Free IAA levels in the transformants are 200–400% higher in young shoot tissues than in the untransformed control, but the levels of cytokinins and cytokinin metabolites are generally lower. The bars for isopentenyladenine and zeatin riboside include isopentenyladenine monophosphate and zeatin ribotide, respectively. (B) Free IAA levels in wild-type and two *ipt*-transformed lines (P3 and P9). The free cytokinin levels in young leaves of these transformants are from 100 to 400% higher for zeatin and more than a 1000-fold higher for zeatin riboside and zeatin ribotide. Free IAA levels, however, are generally reduced. Bars indicate SD. Adapted from Eklöf *et al.* (1997).

root pole. Bilateral symmetry is established with the development of cotyledons. Fry and Wangermann (1976) were the first to state that the initiation of polarized auxin transport in globular embryos might mediate the morphological polarity expressed later in embryogenesis. This concept is supported by experiments with excised embryos treated with inhibitors of polar auxin transport and, more convincingly, by mutants in which such transport is disrupted.

1.1. Embryos Treated with IAA Polar Transport Inhibitors Develop Abnormalities

Surgical experiments in the 1970s with cut hypocotyls of both gymnosperm and angiosperm embryos established that IAA is transported basipetally in embryonic axes and further that this transport is blocked by 2,3,5-triiodobenzoic acid (TIBA). In more recent years, zygotic embryos of a dicot, *Brassica juncea*, and a monocot, wheat, excised at early stages and treated with inhibitors of polar IAA transport [e.g., TIBA, naphthylphthalamic acid (NPA), *trans*-cinnamic acid, or quercetin, see Chapter 13] have been shown to develop abnormalities. The abnormalities include loss of axiality and/or bilateral symmetry, multiple or fused cotyledons, and, in case of wheat, multiple embryos. The effects are dependent on the stage of the embryo at excision and the concentration of the inhibitors used. For instance, embryos of *B. juncea* treated with *trans*-cinnamic acid develop cup-shaped cotyledons, but do not show loss of axiality (Fig. 14-2).



FIGURE 14-2 Scanning electron micrograph of an embryo of Indian mustard (*Brassica juncea*). Embryos were excised at early globular stage and cultured *in vitro* with 80 μ M *trans*-cinnamic acid for 10 days. The embryo shows cup-shaped or fused cotyledons above the hypocotyl, but the root-shoot polarity is not disturbed. Bar: 100 μ m. From Liu *et al.* (1993).

Carrot somatic embryos treated with auxin polar transport inhibitors (TIBA or NPA) show similar abnormal phenotypes. Globular embryos undergo persistent spherical expansion, oblong embryos continue with axis elongation but do not form cotyledons, and heart embryos develop additional axes on their hypocotyls. Further morphogenesis seems to be stopped or altered depending on the stage of excision.

1.2. Mutants Disrupted in Polar Transport of IAA

The discovery of mutants in which polar transport of IAA is disrupted (for *Arabidopsis* mutants, see Table 13-1, Chapter 13) and cloning of their wild-type genes has opened a new chapter in the analysis of pattern-defective mutants in embryogenesis.

As explained in Chapter 13, the *PIN1* gene encodes an auxin efflux carrier that is specifically expressed in shoots (as opposed to *PIN2/EIR1*, which is expressed in roots). The *pin1* mutant has fused cotyledons that appear as a cup on top of the hypocotyl/root axis; thus, the mutant shows loss of bilateral symmetry, but not apical-basal polarity (Fig. 14-3).

In other embryogenesis mutants, apical-basal polarity is lost as well. The *gnom* mutant of *Arabidopsis* has the phenotype of a spherical mass of cells with no polarity, although it can still show tissue differentiation (see Chapter 3). In a remarkable correlation, it has been shown that the *gnom* mutant has a random distribution of PIN1 protein and fails to localize it asymmetrically at the basal plasma membranes of cells, as happens in the wild-type embryos (Fig. 14-4). Thus, the mutant does not develop the capacity for polar auxin transport and does not show apical-basal polarity. The *GNOM* gene encodes a membrane-associated protein, which is thought to regulate vesicle trafficking and which may be associated with the asymmetric localization of PIN1 protein at a very early stage in embryogenesis.

In another mutant of *Arabidopsis*, *monopteros* (*mp*), PIN1 distribution is normal, but pattern formation, as well as vascular development, is affected because axialization of cells in response to auxin cues does not occur (Fig. 14-5). The number of cotyledons can also be affected; instead of the typical two cotyledons, only one or multiple cotyledons may be formed. Thus, the bilateral symmetry of the embryo is also affected. The *MP* gene encodes a transcription factor that is involved in auxin signaling (see Chapter 22). Both *pin1* and *mp* mutants show abnormalities in the adult plants as well, which are traced to a defective polar transport of IAA.

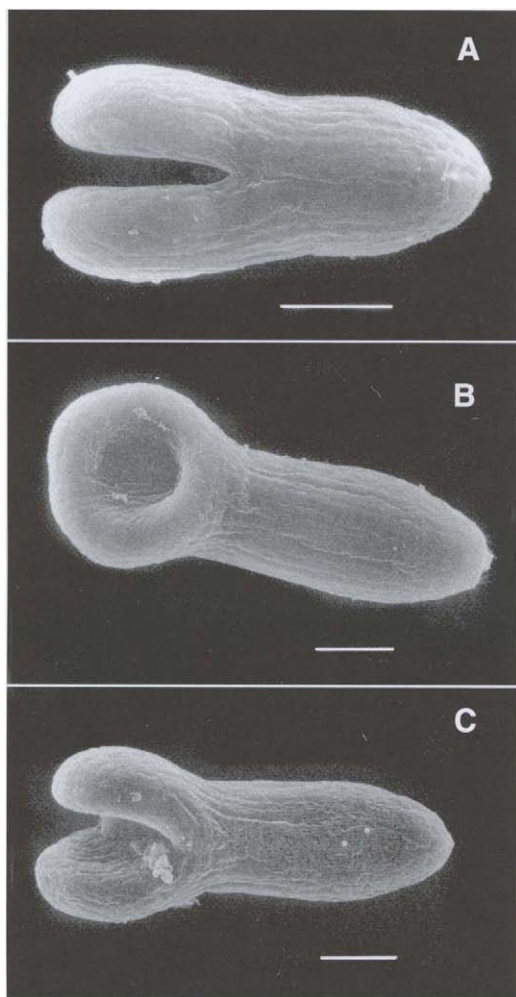


FIGURE 14-3 Scanning electron micrographs of embryos of wild-type and a *pin1* mutant of *Arabidopsis*. Embryos were dissected from selfed heterozygous plants of the *pin1-1* mutant. Three different phenotypes are shown: normal embryo (A), fused cotyledon (B), and intermediate type with partially fused cotyledon (C). Bars: 50 μm . From Liu *et al.* (1993).

More recently, other *Arabidopsis* mutants defective in embryogenesis—*bodenlos* and *axr6*—have been isolated. Both show phenotypes suggestive of defects in auxin perception and/or transport.

Thus, both from a study of mutants and exogenous application of inhibitors of polar IAA transport to zygotic or somatic embryos, it can be concluded that polar transport of IAA has a determining influence on the differentiation of the embryonic axis and on the bilateral symmetry and overall growth pattern of the embryo. As to what specific phenotype is obtained seems to be determined by the site of lesion in polar IAA transport and stage of embryo development at which the lesion becomes effective.

2. INDUCTION OF CELL DIVISION DURING SOMATIC EMBRYOGENESIS

The induction of proembryogenic masses (PEMs) in explants, or suspension cell cultures, from mature tissues usually requires an exposure to auxin, usually 2,4-D, although Dicamba and 1-NAA are also used (see Chapter 3). It is noteworthy that IAA usually gives much poorer results than 2,4-D or 1-NAA, probably because it is subject to metabolism by plant tissues. The duration of exposure to 2,4-D may vary from a few hours to a few days, depending on tissue and its physiological state. The role of auxin seems to be to induce rapid cell division, not to set the single cells on the embryogenic program. The evidence is indirect. Once PEMs have been initiated, it is necessary to remove 2,4-D (or 1-NAA) before embryogenesis can proceed to globular or heart-shaped embryo. In the continued presence of 2,4-D, further embryo development and differentiation are inhibited; instead, the tissues fall back to forming undifferentiated callus or cell proliferation continues without a clear establishment of polarity.

For later stages of embryogenesis, an endogenous supply of IAA is necessary because, as explained earlier, inhibitors of polar IAA transport cause severe abnormalities in the pattern of embryo growth. Also, if auxin homeostasis is disturbed by an exogenous supply of auxins to globular or heart-stage embryos, further development is arrested, while cell proliferation and overall growth continue.

Measurements of endogenous IAA in PEMs in 2,4-D-treated callus of carrot reveal an extraordinarily high concentration of IAA, ca. 60 $\text{ng} \cdot \text{g fw}^{-1}$ free and ca. 600 $\text{ng} \cdot \text{g fw}^{-1}$ total IAA. These high levels are probably necessary for the induction of cell divisions in the hitherto quiescent cells. Later, after transfer to 2,4-D-free medium, the levels decline precipitously. Postglobular embryos contain 15–30 $\text{ng} \cdot \text{g fw}^{-1}$ of total IAA, which is low enough to establish internal gradients and thus polarity. These conclusions are summarized as follows:

[auxin] high \rightarrow cell proliferation and unorganized growth \rightarrow [auxin] reduced \rightarrow auxin gradient established \rightarrow organized growth and development

SECTION III. ROOT AND SHOOT RATIO IN PLANTS

Evidence that endogenous IAA and cytokinin levels are an important factor in regulation of root–shoot

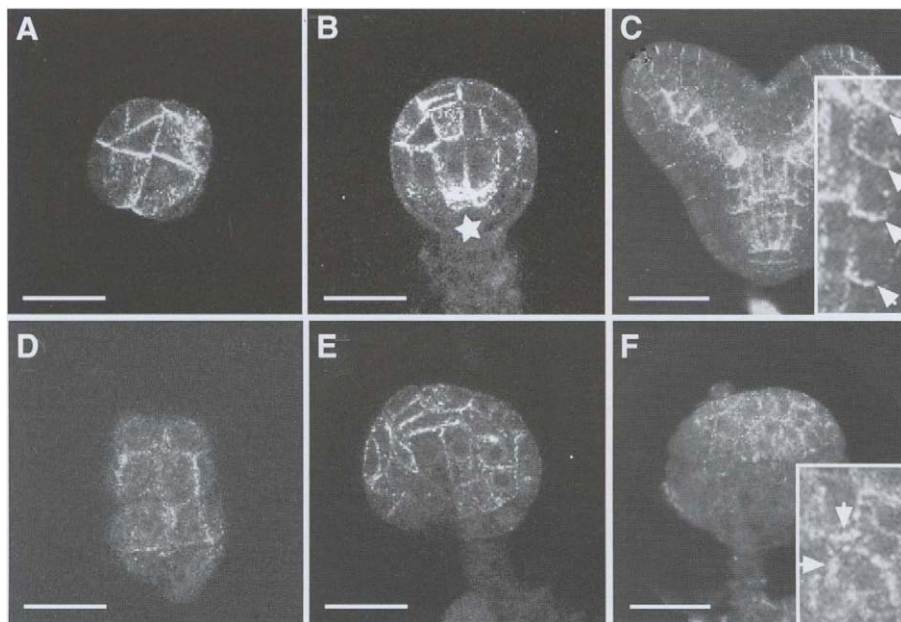


FIGURE 14-4 Immunolocalization of the PIN1 protein in *Arabidopsis* embryos. (A–C) wild type and (D–F), *gnom* mutant. In the wild type, PIN1 shows increasingly polar localization in groups of adjacent cells. Such localization begins as early as the 16 cell stage (A) and is gradually narrowed down to provascular cells, both in the developing cotyledons and in the embryo axis (asterisk in B and arrows in inset in C). In contrast, in the *gnom* mutant, PIN1 localization is disorganized, and cells displaying PIN1 (arrow in inset in F) are not aligned with one another. Bars: 20 μ m. Images were obtained by confocal laser scanning microscopy. With permission from Steinmann *et al.* (1999), © 1999 American Association for the Advancement of Science.

ratios comes from two main sources: tissue culture experiments and transgenic plants overproducing IAA or cytokinins.

1. ROOT-SHOOT RATIOS IN TISSUE CULTURE

When explants taken from plant tissues, pith, cortex, vascular parenchyma, leaf mesophyll, and so on are placed on nutrient-supplemented agar in culture, the parenchyma cells divide for a while and form a callus, but then go moribund and growth more or less ceases. Although such callus tissue can be kept alive in culture almost indefinitely, it requires an exogenous supply of an auxin or an auxin and a CK in appropriate concentrations to initiate cell proliferation and/or tissue/organ differentiation.

Cytokinins are very important for the induction of cell division (see Chapter 15), but calli from most explants can grow successfully on a medium that is supplemented with an exogenous auxin only. The likely explanation is that the explants have a limited supply of endogenous IAA, which gets used up; they cannot synthesize their own IAA, but are able to syn-

thesize CKs. In some cases, the explants are unable to synthesize CKs. For example, in certain lines of tobacco (*Nicotiana* spp.), explants from pith cannot synthesize CKs and require an exogenous supply of a CK to proliferate—they are cytokinin dependent (see Chapter 4). In contrast, cortical explants from the same lines are usually CK autonomous. Callus cultures from other plants and tissues vary in their requirement of these two hormones for proliferation, and these requirements are usually determined by trial and error.

The CK-deficient pith cultures of tobacco have been particularly useful in investigating the effects of exogenously supplied auxin and/or cytokinin on root vs shoot-bud initiation in callus tissues (Fig. 14-6).

It is clear that a higher auxin to CK ratio promotes root formation, whereas a higher CK to auxin ratio promotes shoot-bud formation. These experiments, reported in 1957 by Folke Skoog and Carlos Miller, showed for the first time the intricate balance between IAA and CK ratios in regulating root initiation and bud growth. They have been repeated many times in many other laboratories using different materials and have laid the foundation for the concept that IAA and CKs interact to regulate the root-shoot ratio and many

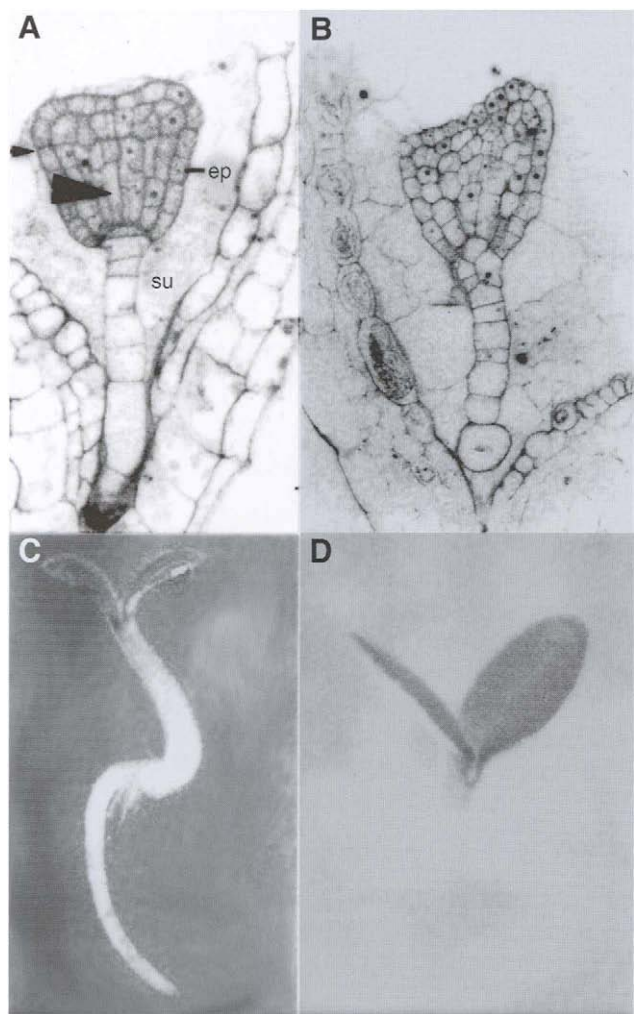


FIGURE 14-5 Wild-type and *monopteros* (*mp*) embryos of *Arabidopsis* at the onset of heart stage (A and B) and as young seedlings (C and D). In the wild-type embryo, files of elongated cells are seen in the subepidermal regions of the incipient hypocotyl/root region (separated from apical region by a small arrowhead), whereas the corresponding cells in the *mp* mutant are neither elongated nor organized in files. The large arrowhead in A points at elongated cells of the incipient vascular system. Note that the suspensor (Su) and epidermis (ep) are not affected. The wild-type seedling consists of a shoot meristem (not visible), two cotyledons, the hypocotyl, and the primary root. In *mp* mutants, cotyledons may be variably fused and the hypocotyl/root region is replaced by a basal peg of unorganized tissue. From Hardtke and Berleth (1998).

other developmental processes in plants. As a practical application, they also provided a technique for generating whole plants from callus cultures. Rooted and budded calli can be transferred to soil and whole plants obtained. Tobacco pith lines that produce endogenous CKs often produce shoot buds only when placed in culture and require exogenous IAA to produce roots.

2. TRANSGENIC PLANTS AND IAA- OR CK-OVERPRODUCING MUTANTS

As mentioned earlier, transgenic plants and mutants overproducing IAA produce far more roots while curtailing shoot production than wild-type plants. Moreover, tissue explants taken from these transformants and placed in culture are prolific in root production and deficient in shoot bud production unless supplied with exogenous CKs. In contrast, plants overproducing cytokinins produce abundant branching and much less or no roots. Tissue explants taken from these plants and placed in culture behave similarly and require exogenous auxins to produce roots.

SECTION IV. HORMONAL REGULATION OF ROOTING

As shown in Chapter 1, lateral roots emerge in the mature region of the primary root. The apical few millimeters are generally free of lateral roots and then their incidence rises to a peak and subsequently falls off (see Fig. 14-9).

Plants also produce “adventitious” roots, i.e., roots initiated at sites other than the root pole of the embryo. Adventitious roots are formed in many plants naturally (e.g., climbing roots in ivy, prop roots in corn, stilt roots in *Pandanus*), as well as in cuttings of stems or leaf petioles, and by “layering.” (In the practice of “layering,” a branch close to the ground is covered by soil and the plant responds by producing roots. The stem segment with the roots is then cut off and propagated as a new plant.) Induction of rooting via cuttings or layering, in addition to propagation by grafting of a scion on to a root stock, are important commercial enterprises, and many horticultural and ornamental plants are propagated by these methods (e.g., azalea, rhododendron, heather, rose, apple). *Chamaecyparis nootkanensis* (yellow cedar) is a valuable timber tree in the north western United States and Canada, but it does not produce a sufficient number of cones and viable seeds. It is also propagated via cuttings or grafting (Fig. 14-7).

Lateral roots and adventitious roots share many common features in their origin and regulation by hormones. In the following section, they are considered together, unless otherwise indicated. It should be noted, however, that adventitious rooting is slightly more complicated because it involves first a dedifferentiation of already committed cells and, in the case of cuttings, has the added dimension of wound response (see Chapter 4).

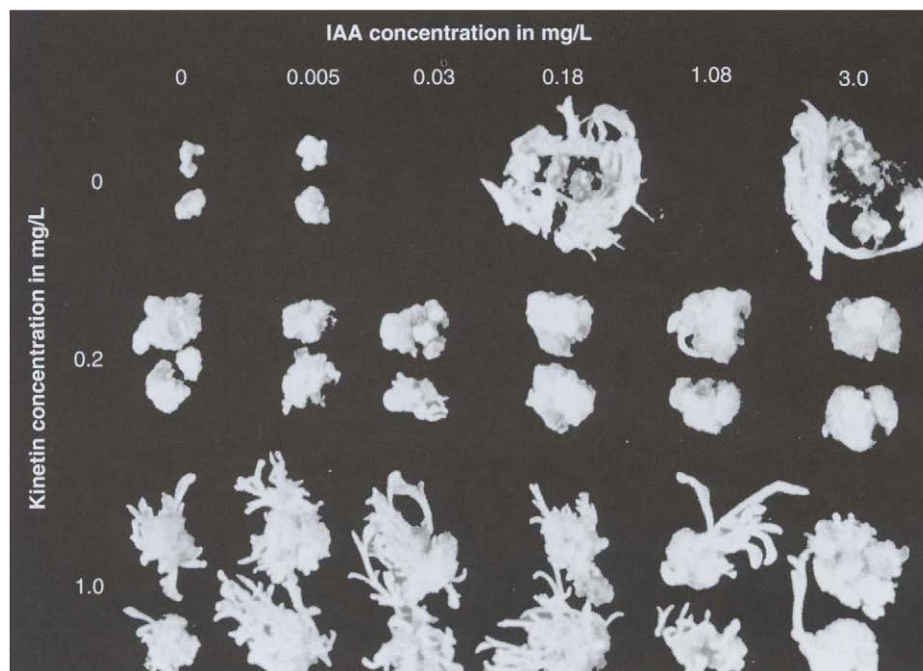


FIGURE 14-6 Initiation of roots and shoot buds in *Nicotiana* pith callus cultures by various concentrations of IAA and CK. Higher concentrations of IAA promote roots to be formed, whereas higher concentrations of cytokinins cause shoot buds to be formed and inhibit root formation. At optimal concentrations of IAA and cytokinins, both roots and shoot buds are formed. From Skoog and Miller (1957).

1. ORIGIN OF LATERAL AND ADVENTITIOUS ROOTS

Lateral root primordia arise by localized cell divisions in the pericycle, resulting in a mound of tissue. Further oriented divisions and cell enlargement in the mound give rise to an organized structure, the root primordium, which acquires its own root apex and root cap and grows through the parent root cortex, finally emerging as a lateral rootlet (Fig. 14-8).

Adventitious roots, likewise, are initiated by cell divisions in the deeper-lying parenchyma cells associated with vascular tissues (stems and leaves of seed plants generally do not have a pericycle). A root primordium is organized similarly and then grows out as a new root.

Formation of a lateral or adventitious root thus has at least three stages: (i) induction of cell divisions in the hitherto "quiescent" pericyclic cells or parenchyma cells of vascular tissues, (ii) organization of a root primordium with its own root apex, root cap, and histological zonation, and (iii) growth of the primordium and its emergence as a lateral root. As in other developmental phenomena, these stages, especially the latter two, are not sharply delineated and partially overlap.

2. ROOT FORMATION IS STIMULATED BY AUXINS AND INHIBITED BY CYTOKININS

From the earliest days of auxin research, auxins, both natural [IAA, 4-chloro-indoleacetic acid, indolebutyric acid (IBA), phenyl acetic acid (PAA)] and synthetic (1-naphthalene acetic acid or 1-NAA), have been known to stimulate the formation of lateral and adventitious roots. Interestingly, IAA, at the time of its discovery in 1930s, was known as the "rooting hormone," a designation now used for IBA and its commercial preparations.

Several lines of evidence indicate that while auxins stimulate root formation, cytokinins inhibit it.

2.1. Surgical Removal of Sites of IAA or Cytokinin Biosynthesis

A plot of the distribution of lateral root primordia and emerged lateral roots is shown in Fig. 14-9. It is evident that the apical 5-mm segment lacks primordia and that the incidence of primordia rises to a maximum level before falling gradually to zero. The emergence of lateral roots follows a similar pattern but with a delay. In a seedling, cotyledons and epicotyl are the



FIGURE 14-7 Propagation of *Chamaecyparis nootkanensis* by cutting. This plant, now about 2-years old, was started from a cutting about the size indicated by the scale. Rooting takes about 3–6 months depending upon where in the plant the cutting comes from and time of the year when the cutting is taken. Usually, young plants are better sources than old plants and spring-early summer are the best times in the year. Courtesy of Bev Greenwell, Happy Hollow Nursery, Abbotsford, B.C.

major source of IAA. Their removal severely reduces the number of lateral root primordia formed. In contrast, if the tips of primary roots are decapitated, there is a sudden although transitory increase in the production of lateral primordia, presumably because of loss of the cytokinin source and an increase in the IAA/CK ratio at the cut end. These results correlating the presence of an endogenous auxin source or removal of root tip (cytokinin source) and rooting have been reported in many studies.

2.2. Application of Auxins and Cytokinins

If natural or synthetic auxins are supplied to intact roots or cuttings, the induction of roots is stimulated greatly. In a study using lettuce seedlings, 1-NAA supplied to the primary roots increased the number of lateral root primordia by 600% over controls (Fig. 14-10). This induction by NAA was inhibited by an application of a cytokinin, kinetin.

The effect of cytokinins on rooting, however, varies with concentration as well as duration of treatment. Higher concentrations and longer treatments are inhibitory, whereas lower concentrations and shorter treatment times may have an enhancement effect. Table 14-1 shows the effects of benzyladenine (BA) on rooting in pea stem cuttings. BA at concentrations of 10^{-7} M or higher inhibited root primordia formation. Lower concentrations given for 4 days or longer also inhibited primordia formation, but if given only for 1 or 2 days had a promotive effect. Similar results were obtained with zeatin.

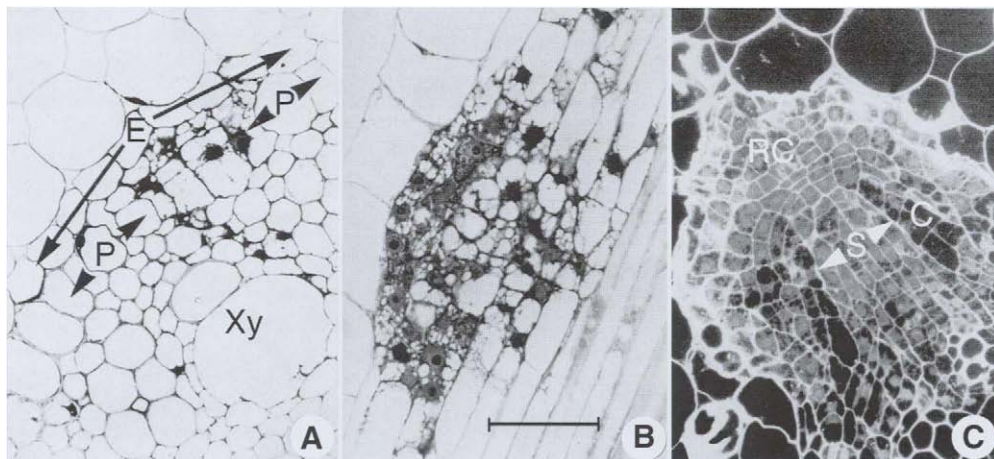


FIGURE 14-8 Development of a lateral root in *Zea mays*. (A) A transverse section of the parent root showing an early stage. Pericline and anticline divisions in the pericycle (P) opposite a xylem pole (Xy) herald the development of a lateral primordium. Arrowheads indicate the extent of pericycle tissue involved. An endodermis (E) is indicated by arrows. External to it are large cortical cells. (B) A longitudinal section of

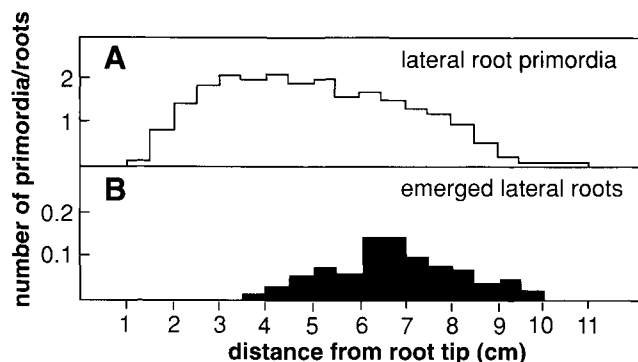


FIGURE 14-9 Distribution of lateral root primordia (A) and emerged lateral roots (B) along the primary root of 3-day-old pea (*Pisum sativum*) seedlings. Lateral root primordia were visualized by clearing of roots in 5% chromic acid for 72 h. From Wightman and Thimann (1980).

In the study just described no exogenous auxin was supplied. In auxin-treated material, the negative effect of CKs is most pronounced if they are given at a sufficiently high concentration (5×10^{-7} M or higher), either simultaneously or soon after the auxin treatment period during which root primordia are initiated. Figure 14-11 shows such inhibition by 24-h pulses of BA given to apple microcuttings at different times from 1 to 6 days. In this case, maximal inhibition occurred if the BA treatment was given at 48 h. It is also clear that if CK treatment is delayed past a certain time, there may be no inhibition.

Data from adventitious rooting indicate that the inhibition by CKs is dependent both on concentration of the CK used and on the specific time and duration of application.

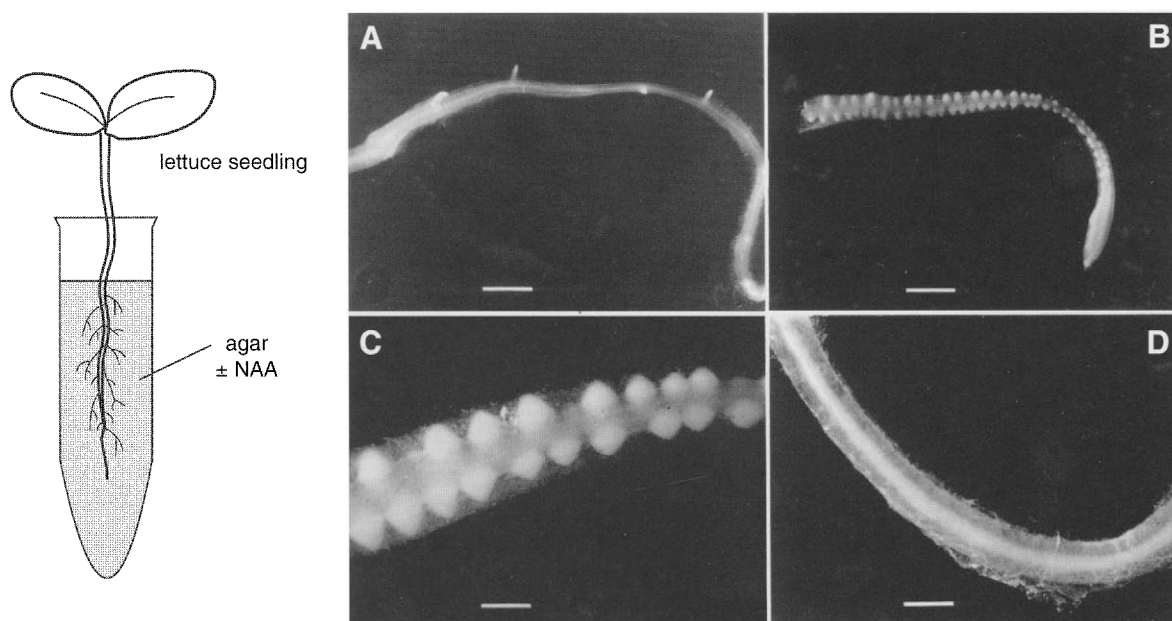


FIGURE 14-10 Induction of lateral roots in lettuce (*Lactuca sativa*) seedlings. Lettuce seedlings with 10-mm-long roots were transferred to micromold capsules containing 0.6% agar with or without NAA (see schematic on the left), and the induction of lateral roots was monitored after 72 h. To obtain the photographs, roots were cleared in 3% chromic acid. Bar: 5 mm. (A) Root grown in agar (control). (B) Root grown in NAA (10^{-5} M) for 72 h. (C) An enlargement of a portion of the root in B. (D) A segment of a root grown in NAA (10^{-5} M) and kinetin (2×10^{-5} M). From MacIsacs *et al.* (1989).

FIGURE 14-8 (Continued)

the parent root through the center of a lateral primordium at a later stage. Cell divisions in the pericycle have given rise to a mound of tissue composed of small, densely cytoplasmic cells. An endodermis is seen external to it. (C) A transverse section of the parent root through the center of a lateral primordium at a still later stage. The primordium is about half-way through the parent cortex; it has acquired its own root apex and root cap (RC). Provascular tissue (stele, S) and cortex (C) are also demarcated. The lateral primordium is growing toward the top left corner. Bar: 50 μ m. From Bell and McCully (1970).

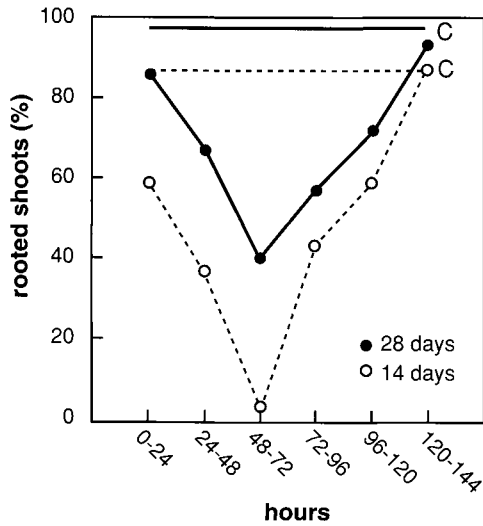


FIGURE 14-11 Inhibition of rooting in apple microcuttings by BA. Shoot cuttings were cultured on medium with $1\ \mu\text{M}$ IBA and received a 24-h pulse with $1\ \mu\text{M}$ BA at the indicated times. Roots were counted after 14 and 28 days. C, controls that did not receive any BA treatment. From de Klerk *et al.* (1995).

2.3. Mutants and Transgenics That Overproduce IAA or Cytokinins

Results from the surgical removal of sites of IAA or CK biosynthesis and from the exogenous application of auxin or CKs to whole plants or stem cuttings are supported by mutants and transgenics that overproduce IAA or CK. The *rooty* mutants of *Arabidopsis* (see Table 6-2) have much higher levels of endogenous free IAA

TABLE 14-1 Influence of Benzyladenine (BA) on Rooting^a

BA concentration (M)	Duration of BA treatment (days)			
	1	2	4	11
3×10^{-9}	131 ± 6	110 ± 6	90 ± 6	69 ± 5
10^{-8}	106 ± 6	101 ± 6	53 ± 7	6 ± 2
10^{-7}	81 ± 5	37 ± 6	0	0
10^{-6}	20 ± 4	0	0	0

^aPea (*Pisum sativum*) stem cuttings were kept in modified Hoagland solution; BA was present in the nutrient medium from the start of the experiment and for the number of days indicated. Roots were counted after 11 days. Mean number of roots per cutting is expressed as percentage of control \pm SE for 30 cuttings. Mean number of roots formed in control cuttings was 19 ± 1 . From Bollmark and Eliasson (1986).

and IAA conjugates than the wild-type plants and produce abundant lateral and adventitious roots (Fig. 14-12). The *ROOTY* gene encodes a protein with similarity to tyrosine aminotransferases, but it is not clear how its loss of function brings about elevated levels of IAA.

Likewise, transgenic plants overexpressing *iaaM* *iaaH* genes produce abundant roots. In contrast, the cytokinin overproducing *Arabidopsis* mutant *amp1* and transgenic plants overexpressing the *ipt* gene for cytokinin biosynthesis show a curtailment in the production of lateral roots and, in extreme cases, even a lack of all laterals and root growth. Application of cytokinins to *rooty* mutants results in a curtailment of lateral root production.

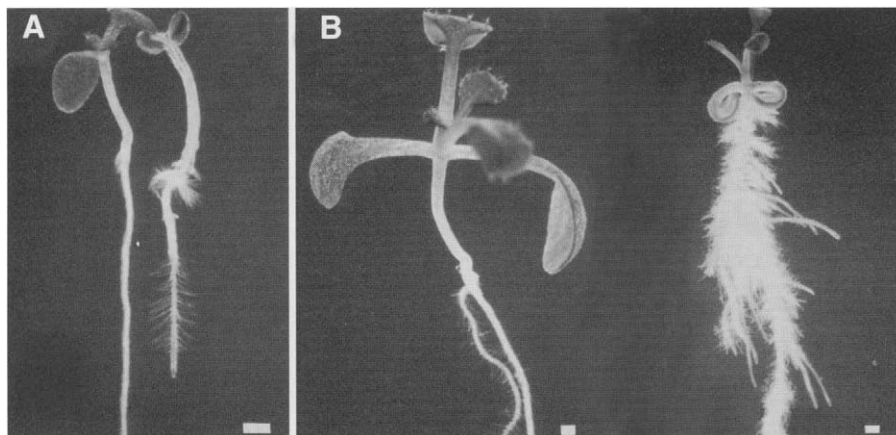


FIGURE 14-12 Wild-type (left) and *rooty* mutant (right) of *Arabidopsis*. (A) Seedlings after 6 days in light and (B) after 12 days in light. From King *et al.* (1995).

That endogenous auxins (IAA or IBA) play an important role in lateral root formation is reinforced by the fact that mutants inhibited in the polar transport of IAA (e.g., *aux1*, see Chapter 13) or insensitive to auxins (e.g., *axr1*, *axr2* mutants of *Arabidopsis* and *diageotropica* mutant of tomato, see Chapters 22 and 27) are also deficient in the production of lateral roots. Evidence from mutants in auxin transport or sensitivity is corroborated by evidence from the application of auxin transport inhibitors, such as triiodobenzoic acid (TIBA) or naphthylphthalamic acid (NPA). Application of these inhibitors to stems inhibits lateral rooting basipetal to the site of application. Applied to roots, they inhibit lateral rooting distal to the site of application (i.e., toward the root tip). Likewise, application of inhibitors of auxin action [e.g., α -(p-chlorophenoxy) isobutyric acid (PCIB), phenoxyacetic acid (POA)] is reported to inhibit adventitious rooting in poplar shoots.

2.4. IAA Transported Acropetally in Roots Seems To Be Involved in Induction of Lateral Roots

In roots, IAA is transported both acropetally in the vascular cylinder (phloem or precursor procambial cells) and basipetally from the root tip toward the root-shoot junction (see Fig. 13-10). The question arises whether IAA transported acropetally or basipetally plays a role in the induction of lateral roots. The combined evidence from several sources suggests strongly that IAA coming down from the shoot and transported acropetally in the central cylinder plays a role in the induction of lateral roots whereas that transported basipetally plays a role in tropic responses. For instance, the agravitropic mutants of *Arabidopsis* (*eir1/pin2*) are inhibited in basipetal IAA transport, but are normal in lateral root production. Localized application of NPA at the root-shoot junction reduces the total IAA content in the root and also results in a reduced number of lateral roots formed (Table 14-2).

Finally, in a study in which radiolabeled IAA was fed to shoot tissue of pea seedlings, the radiolabel accumulated at selected sites in the root, sites presumed to be the initiation sites for lateral roots (Fig. 14-13).

3. ROOT INITIATION AND ROOT GROWTH HAVE DIFFERENT AUXIN CONCENTRATION OPTIMA

It is a curious fact that while auxins promote initiation of lateral roots, no sooner are such roots initiated

TABLE 14-2 Effect of localized NPA Application at the Root-Shoot Junction on Free IAA Concentration^a

Treatment	Free IAA concentration (ng/g fresh weight)	Lateral root number
Control agar	25.8 + / - 4.7	15.5 + / - 0.8
NPA agar	17.6 + / - 2.2	5.9 + / - 0.5

^aSeven- or 8-day-old seedlings of *Arabidopsis* were treated by application of agar with or without NPA at the root-shoot junction. After 3 additional days of growth, roots were harvested and their IAA concentration was determined by GC-MS or the number of lateral roots was counted. The reduction in free IAA concentration or the difference in root number was statistically significant. From Reed *et al.* (1998).

than their growth is inhibited by auxins. In fact, root-growth of laterals, as well as main roots, is inhibited under all except very low auxin concentrations (usually 10^{-9} M or below). However, for induction of lateral (or adventitious) roots, concentrations of 10^{-6} to 10^{-5} M are required, a difference in concentration of 3-4 orders of magnitude (Fig. 14-14).

How such sharp concentration differences are obtained at specific sites in an intact root is not known. One can speculate that localized cells in pericycle accumulate IAA while it is being transported in the vascular cylinder and that it is these cells (or groups of "founder" cells) that serve as progenitors

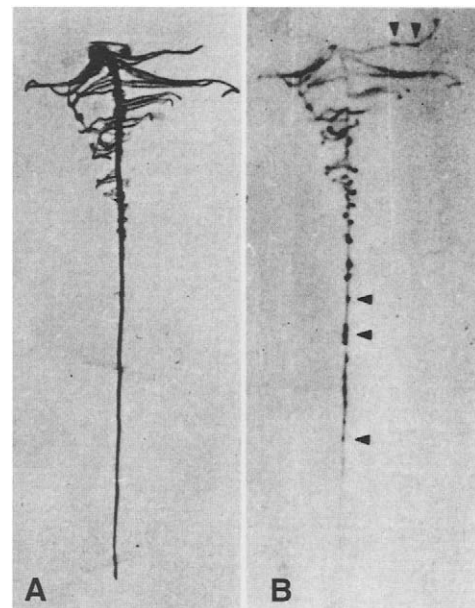


FIGURE 14-13 Transport of IAA in pea seedlings. [¹⁴C]IAA was applied to the shoot apex, and the radiolabel was localized after a 24-h transport by autoradiography. Radiolabel can be seen accumulated at localized sites in the root, sites believed to represent lateral root primordia. From Rowntree and Morris (1979).

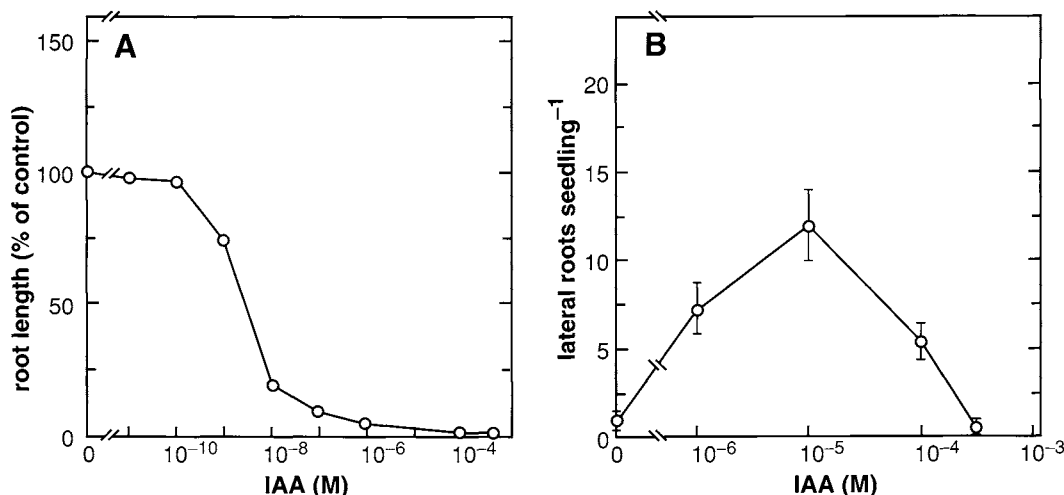


FIGURE 14-14 IAA dose-response curves for root growth and production of lateral roots in *Arabidopsis*. (A) Seedlings were germinated and grown in darkness for 5 days in the presence of IAA. Root length is expressed as a percentage of the IAA-free control (100% = 10.5 ± 0.2 mm). (B) Seedlings were germinated and grown in darkness for 3 days and then transferred to media containing IAA. Data are the means \pm SE for the number of roots per seedling 5 days after transfer. Adapted from Knee and Hangarter (1996).

to lateral root primordia. Such a localized accumulation of IAA seems to be supported by the study given earlier in which radiolabeled IAA was fed to shoot tissue of pea seedlings (see Fig. 14-13).

4. ETHYLENE MAY BE INVOLVED IN INDUCTION OF ROOTING

There are conflicting reports whether ethylene promotes adventitious rooting in stems or stems cuttings. It seems that ethylene has a promotive effect only in the presence of an auxin source. Thus, in sunflower hypocotyl cuttings, application of ethylene-producing compounds such as ACC or Ethephon, by themselves, had no effect on rooting, but in the presence of an endogenous auxin source (cotyledons or apical bud) or applied auxin had a stimulatory effect. Application of inhibitors of ethylene biosynthesis (e.g., aminoethoxyvinylglycine) or ethylene action (e.g., silver thiosulfate or 2,5-norbornadiene) also reduces the number of adventitious roots formed.

Many plants growing in wetlands (e.g., *Rumex palustris*) are subjected to periodical flooding, which causes a deficiency of oxygen (hypoxia) in roots. The flooded plants respond by producing adventitious roots higher up in the stem tissues. The signal of flooding can be duplicated by growing plants or placing stem cuttings in deoxygenated agar. In these cases, it has been shown that rooting is obtained *via* an interaction between

ethylene and endogenous IAA. That both hormones are involved is shown by the fact that inhibitors of ethylene biosynthesis cause an inhibition of rooting; likewise, application of inhibitors of polar auxin transport, such as NPA, also inhibits rooting. Flooding results in an accumulation of ethylene that cannot diffuse out of root tissues because the solubility of ethylene in water is much lower than in air. It is thought that the increased concentration of ethylene, in turn, alters the sensitivity of tissues to endogenous auxin and that auxin is the primary rooting hormone.

That ethylene is involved in rooting is also indicated by a mutant of tomato, *never-ripe*, which is insensitive to ethylene in fruit ripening. Stem cuttings from this mutant produce far fewer adventitious roots when treated with auxin (IBA) than stem cuttings from the wild-type tomato, which suggests that the promotive effect of auxin on adventitious rooting is influenced by ethylene responsiveness.

Auxin is a known inducer of ethylene biosynthesis (see Chapter 11), but the nature of the auxin-ethylene interaction in the control of rooting is still obscure.

5. MUTANTS DEFECTIVE IN LATERAL ROOT FORMATION

To dissect the *in planta* role of IAA in lateral root formation, *alf* (for aberrant lateral root formation) mutants of *Arabidopsis* were obtained. Three single gene recessive mutations at distinct loci were identified:

alf1-1, *alf3-1*, and *alf4-1*. Among these, *alf1-1* is an over-producer of IAA and is allelic to the *rooty* mutants (see Table 6-2), whereas *alf4-1* does not produce any laterals and is not rescued by IAA. The *alf3-1* mutant produces aberrant laterals—they remain arrested as a bolus of undifferentiated cells and such boli pile up upon each other instead of arising at some distance from each other and completing to mature organized structures (Fig. 14-15). *alf3-1* mutants are rescued by indole as well as IAA, and also partially by crossing with *alf1-1* (which overproduces IAA). The number of pericycle cells or position at which laterals arise is not changed in either *alf1-1* or *alf3-1*.

The *ALF3* gene has not been cloned, and the nature of its gene product is unknown. However, these data, while confirming that IAA is necessary for initiating cell divisions in the pericycle, indicate a dual role for the *ALF3* protein in coordinating cell division in the pericycle and subsequent organization and growth of the lateral root primordium.

6. MOLECULAR MARKERS FOR LATERAL ROOT INITIATION AND DEVELOPMENT

Although the pericycle is the seat where lateral root primordia (LRP) are initiated, and pericycle cells that become the “founder” cells are usually localized opposite the xylem poles, it is not known which pericycle cells will initiate the cell proliferation process. Little is known also about early organization and cell specification in a LRP. Similarly, which vascular parenchyma cells will initiate adventitious root primordia or how the adventitious root primordium becomes organized is unknown. These questions are of importance in deciphering the roles of hormones in the initiation and development of lateral/adventitious roots.

Some years ago, a gene from tobacco (*Nicotiana tabacum*), *HRGPnt3*, which encodes the protein moiety of a hydroxyproline-rich glycoprotein (HRGP), was found to be selectively expressed in pericyclic cells forming lateral root primordia. The gene was also expressed in vascular parenchyma cells in stem cuttings, which formed adventitious roots. Unfortunately, there has been little follow-up, and the function of a hydroxyproline-rich glycoprotein in root initiation is not known.

More recently, detailed studies in *Arabidopsis* root indicate that LRP development proceeds in a highly organized manner by a combination of anticlinal and periclinal cell divisions and differential cell enlargement, accompanied by cell and tissue specification. A number of cell/tissue marker lines were generated by fusing the coding sequence of the β -glucuronidase (*GUS*) gene to a minimal CaMV35S promoter and using the construct to transform *Arabidopsis* roots via *Agrobacterium*. The insertions are random, but they can promote expression of a neighboring gene, which is monitored by GUS staining. Although the identity of the gene remains unknown, such expression of *GUS* occurs in a cell type-specific manner and can be used to follow the fate of a specific cell/tissue type. Several marker lines specific for a cell/tissue type were obtained in this manner (Fig. 14-16). Use of these marker lines indicates that tissue layers, such as epidermis, cortex, endodermis, and stele, become non-identical, i.e., they are specified, very early in the development of the LRP, at a stage when a defined root apical meristem is still lacking. This means that the early growth of the LRP, following cell proliferation and tissue specification, does not derive from an apical root meristem (i.e., is with “stem” cells). The root meristem becomes distinct and functional on emergence of the lateral root.

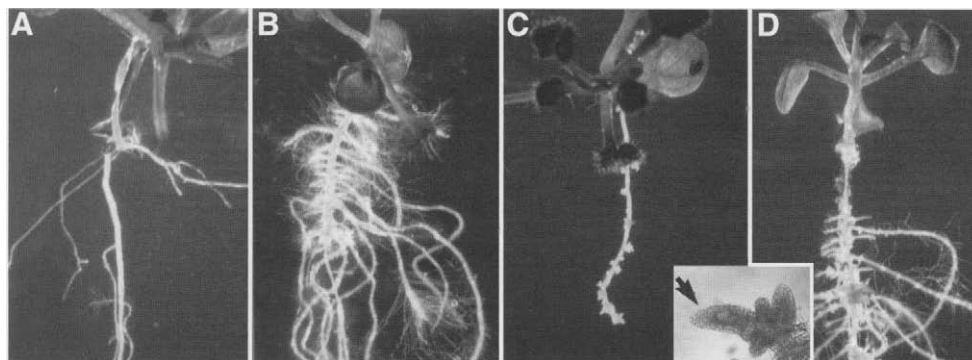


FIGURE 14-15 Wild-type and two aberrant lateral root formation (*alf*) mutants in *Arabidopsis*. (A) Wild type, (B) *alf1-1*, (C) *alf3-1*, and (D) *alf1-1 alf3-1* double mutant phenotypes. (Inset) Part of the *alf3-1* root, showing knob upon knob of laterals; arrow points to a partly elongated lateral root. Plants were photographed under dark-field illumination at 16 days after germination. From Celenza *et al.* (1995).

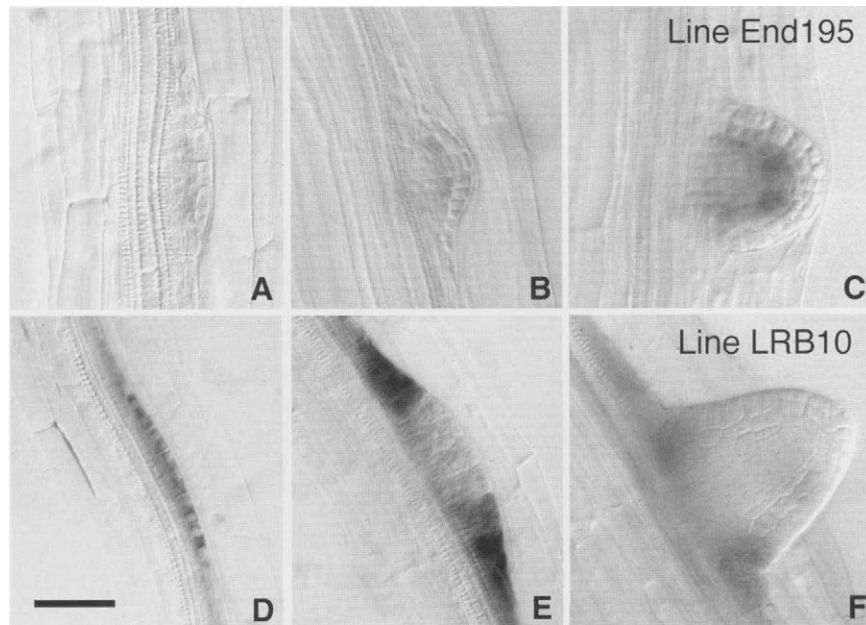


FIGURE 14-16 GUS expression of two cell/tissue-specific marker lines, line End195 and line LRB10, in developing lateral root primordia (LRP) of *Arabidopsis*. LRP development is arbitrarily divided into eight stages, where stage I corresponds to the first sign of LRP formation by the onset of anticlinal and periclinal divisions in localized areas of pericycle and stage VIII corresponds to emergence. Nomarski images of cleared whole mounts of LRPs are shown. (A–C) Line End195, specific to endodermis. Staining starts at stage V when the LRP is still only four cell layered. In (C) staining is occurring in a file of cells seen in surface view. (D–F) Line LRB10, one of the lines expressed very early (stage I) in LRP development. At this stage it is expressed throughout the primordial cells; later the expression becomes progressively restricted to the periphery, but remains as a ring around the base of the emerged lateral root (stage VIII). Bar: $\sim 50 \mu\text{m}$. From Malamy and Benfey (1997).

7. COMMERCIAL APPLICATION OF AUXINS IN ROOTING

Induction of adventitious rooting in stem or leaf cuttings is of considerable commercial importance for the propagation of plants by asexual means.

Different species and cultivars differ in their propensity to form adventitious roots. Some species and cultivars root easily, without any exogenous auxin treatment, and this trait has been correlated to a high endogenous IAA content, whereas others root with difficulty and have a correspondingly low IAA content. Genetic studies correlating rooting capacity to specific genes are rare. In a study on *Arabidopsis* ecotypes and inbred lines, the rooting response by seedling hypocotyls to applied auxin varied from a mean root count of 23.1 (high) to 1.7 (low). Genetic analysis further indicated that high and low rooting responses were probably controlled by multiple genes.

Age-related differences also occur. Stem cuttings from young shoots with a few leaves root with greater ease than older twigs with mature leaves, possibly because the former have more endogenous IAA.

Among the various auxins, IAA, IBA, and 1-NAA, all induce adventitious rooting. IBA is the most widely used auxin and, as mentioned earlier, IBA or its commercial preparation has come to be known as the “rooting hormone.” At similar concentrations, IBA and 1-NAA are usually more effective than IAA. As shown in Fig. 14-17, in apple microcuttings, maximum initiation of roots occurred at 3.2 to 10.0 μM IAA or IBA, but IBA produced 50% more roots than IAA. Reasons for the greater potency of IBA are unclear. It could be that IBA is more stable than IAA because the longer side chain is more resistant to oxidation; thus, it may show greater activity at similar concentrations. It is also interconvertible with IAA *in vivo* (see Chapter 6), although it seems unlikely that the converted form induces rooting. The greater activity of 1-NAA may be related to its greater metabolic stability in plant

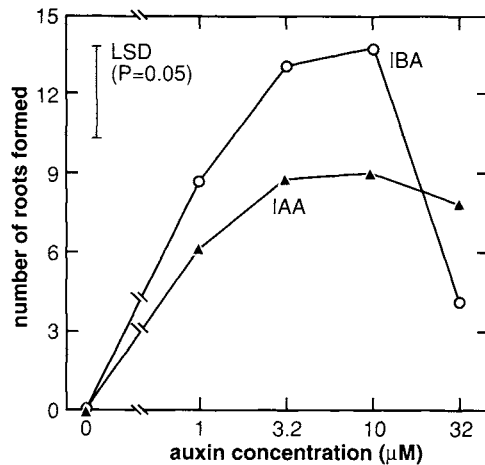


FIGURE 14-17 Effects of IBA and IAA on adventitious rooting in apple (*Malus*) shoot cuttings. Cut shoots were incubated for 5 days in the dark on media with different IBA or IAA concentrations. Roots were counted 21 days after placement in light. Values are means of 20 cuttings and are expressed as roots per shoot. The vertical bar represents LSD ($P = 0.05$). From Van der Kriecken *et al.* (1992).

tissues, but, commercially, it has found much less use than IBA and, in some plant varieties, may even inhibit root initiation.

Treatment with auxin is usually limited to a short duration, from a few hours to a few days, possibly because of the inhibitory effect of auxins on root growth, but the interesting thing is that the induction and emergence of roots may not occur for weeks or months after the auxin treatment. Conifers are generally difficult to root plants. Kenneth Thimann, a pioneer and the dean of auxin research in North America for more than 40 years, recorded a case of Canada hemlock (*Tsuga canadensis*) twigs that were treated with IAA for 24 h and then placed in potting soil. The twigs were examined periodically and replaced. The first roots did not appear until after 3 months (Thimann, 1977). What happens during this long interval is a mystery.

The time of the year when cuttings are taken also seems to have an effect on the ease of rooting. In temperate climates, cuttings taken in spring or early summer seem to root more easily than those taken in the fall or winter. Some studies have also shown that there is a specific period after cutting when the tissue is most responsive to applied auxin. Thus, in microcuttings of apple (*Malus*), auxins were most effective if applied as a 24-h pulse after cutting; the response declined to much lower levels 4 days after cutting (Fig. 14-18).

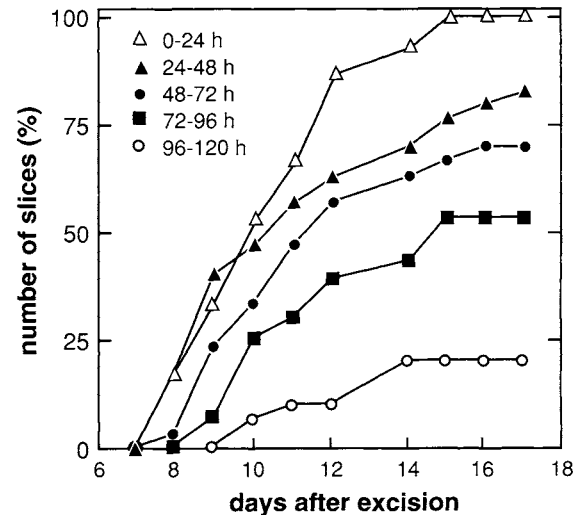


FIGURE 14-18 Sensitivity of apple microcuttings to applied auxin. One-millimeter slices from stems of apple were cultured on a mesh in a petri dish on IBA-free medium and received a 24-h pulse with 25 μM IBA at the indicated times. The number of slices with roots was determined every day. From de Klerk *et al.* (1995).

8. SECTION SUMMARY

Lateral and adventitious roots arise by cell divisions in localized areas in the pericycle in the root and vascular parenchyma cells in stem or leaf tissues. Cell divisions lead to a small protuberance, which organizes itself into a root primordium and grows through the parent root or stem tissue to emerge as a new root. Auxins are the principal hormone that initiate rooting. Evidence comes from several sources: exogenous application of auxins to intact roots or stem cuttings, removal of the endogenous auxin source, and mutants and transgenic plants that overproduce IAA. Significantly, the concentrations of auxins that initiate cell divisions as a prelude to rooting are 3–4 orders of magnitude higher than those that allow root growth. Initiation of roots and their subsequent growth thus involve a very precise adjustment in concentrations of free IAA in root cells. Cytokinins inhibit initiation of rooting by auxins, although exactly how such inhibition is obtained is not clear. Cytokinins inhibit root growth also. There is substantial evidence that ethylene interacts with auxin to control adventitious rooting in stems or stem cuttings. Cell- and tissue-specific cell lines are being used to determine the early stages of lateral root initiation and development, and may prove useful in identifying which pericyclic cells become potentiated to develop as root primordia and the specific roles of hormones in control of such potentiation and subsequent development. In this endeavor,

an *Arabidopsis* mutant with an altered root morphology, where the coordination between cell divisions and organization of root primordium and growth seems to be lost, may also prove useful. Auxins, especially IBA, are important commercially for the induction of rooting in many horticultural and ornamental plants.

SECTION V. APICAL DOMINANCE

Apical dominance is defined as an inhibitory control exercised by the apical portions of the shoot over the growth of the lateral buds below. The phenomenon is easily demonstrated by decapitation. If the main vegetative shoot apex of a plant such as *Helianthus* (sunflower) is cut off, inhibition is released and the buds below respond by growing out (Fig. 14-19). Indeed, any mechanical wounding or chemical treatment that injures the main apex causes lateral buds to grow.

1. APICAL DOMINANCE IS A COMPLICATED PHENOMENON

Even though apical dominance can be defined in simple terms, it is a complicated process in which the genetic makeup of the plant, environmental factors,

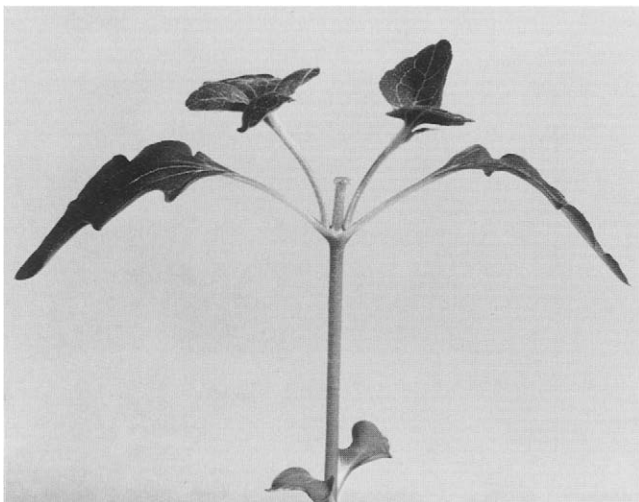


FIGURE 14-19 Apical dominance in *Helianthus annuus* (mammoth gray-striped sunflower). Plant showing lateral bud outgrowth approximately 1 week after decapitation. Courtesy of Morris Cline, Ohio State University, Columbus, OH.

and developmental and hormonal signals all play a part. Most interestingly, although apical dominance is a phenomenon typically seen in shoots, roots have an important role in its regulation.

Apical dominance determines the branching pattern and the shape of plants, but it does so not by regulating the initiation of lateral buds, but whether they subsequently grow out. Initiation of lateral buds, as explained in Chapter 1, is related to the activity of the shoot apical meristem and has little to do with their subsequent growth. Apical dominance is best seen in herbaceous plants and in trees in their first year of growth. In older trees, the phenomenon becomes more complex, being influenced by shading and resource allocation, but can still be seen in branches in their current years' growth. Apical dominance has been better studied in herbaceous plants, and, unless otherwise stated, the following account pertains mostly to them.

The state of arrest or quiescence of lateral buds is not to be confused with bud "dormancy," which has an entirely different physiological basis. Bud dormancy applies to terminal as well as lateral buds and, in plants native to temperate and colder regions of earth, is regulated by temperature and possibly photoperiod.

1.1. Apical Dominance Varies with Species and Cultivars

Apical dominance is genetically based and varies with species and cultivars. Some species show strong apical dominance [e.g., *Helianthus annuus* (sunflower), *Tradescantia* sp.]. Field-grown sunflower plants, 4 feet tall, may show little or no lateral branching in intact plants. In a nursery or greenhouse, by suitable gibberellin treatment, they can be made to grow 7–8 feet tall and still show no branching. Other species show an intermediate or partial apical dominance, i.e., there is some branching [e.g., *Phaseolus vulgaris* (common bean), *Vicia faba* (broad bean), *Pisum sativum* (pea), *Ipomoea nil* (Japanese morning glory)], whereas still others show very weak or no apical dominance—they show substantial and continuing branching in intact plants (e.g., *Coleus*, *Arabidopsis*).

1.2. Environmental Factors Affect Apical Dominance

Various environmental factors, such as availability of nutrients, proximity of neighbors, light quantity, and length of photoperiod, regulate bud outgrowth and affect apical dominance. Experiments with *Coleus*, a plant with weak apical dominance, show that plants grown under low light conditions are much less



FIGURE 14-20 *Coleus* plants grown under low light indoors (left) and under high-light intensity in a greenhouse (right). With permission from Cline (1996).

branched, whereas those grown under high irradiance are branched and bushier (Fig. 14-20).

Experiments with tall varieties of pea plants show that short photoperiods cause them to be bushier and more branched than long photoperiods. In pea, long photoperiods also promote a change from vegetative growth to flowering.

1.3. Apical Dominance Changes with Developmental Status

Several developmental cues, including distance of the lateral bud from the terminal apex and a change to reproductive phase, affect bud outgrowth. In herbaceous forms with strong to moderate apical dominance, inhibition is greater for the lateral buds closer to the apex. As the distance from the apex increases, inhibition lessens and lateral buds are released from dominance. Thus, branching becomes more common in lower internodes. In contrast, in some other plants, the lower, older nodes may remain free of branching, whereas the younger nodes may show lateral outgrowths. Young trees of many species, e.g., *Fraxinus* (ash), *Acer* (sycamore), *Alnus* (alder), and *Ginkgo* (a gymnosperm), show strong apical dominance, but as the tree ages, lateral buds begin to grow and the tree becomes much more branched.

The change to the reproductive phase brings about major changes in branching patterns in many plants. For example, lateral buds that were inhibited from growing in the vegetative phase may be released from inhibition with the onset of flowering (e.g., oat, *Perilla*). Fruiting and seed set may reimpose the inhibition (e.g., *Phaseolus*).

2. GROWTH OF LATERAL BUDS

Lateral buds are held in a state of quiescence until their release from inhibition, and the question arises what happens on decapitation (or other injury to main apex) that induces them to grow. It is useful to distinguish between the actual release from inhibition and the events leading to growth of the lateral bud, but in practice such distinction is not easily attained. While there is little information on what constitutes the “release” of buds from quiescence, bud growth following decapitation can occur very quickly and involves both cell division and cell enlargement. Bud elongation in pea, bean, and many other plants may be seen within 3–10 h after decapitation, but in some species with strong apical dominance, such as sunflower, it may take much longer. Most of the early growth is due to cell enlargement, but mitotic activity starts very quickly. In bean with medium apical dominance, cell growth may resume within 4 h, and mitotic activity within 24 h after decapitation; in contrast, in *Tradescantia*, with strong apical dominance, these processes take much longer. The nuclei in lateral buds are arrested in G1 or G2 of the cell cycle and, among species with similar apical dominance, at least some differences in timing of mitosis are due to the relative proportions of cells arrested in G1 or in G2.

The early molecular events that follow decapitation are unknown. Beginnings are being made with some systems that can be manipulated experimentally, such as pea (*Pisum sativum*). In pea plants, three to four lateral buds occur at each node beneath the terminal apex (Fig. 14-21). Decapitation leads to growth of all of them, but very soon, one lateral becomes dominant and others go into quiescence, although they remain metabolically active. If the newly dominant apex is

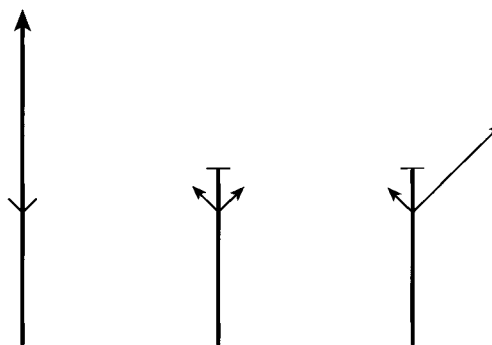


FIGURE 14-21 Schematic drawing of lateral bud growth in pea (*Pisum sativum*) following decapitation of a terminal bud. Only two lateral buds are shown. In the middle diagram, both buds have begun to grow after decapitation of the terminal bud; in the diagram on the far right, one of the laterals has assumed dominance.

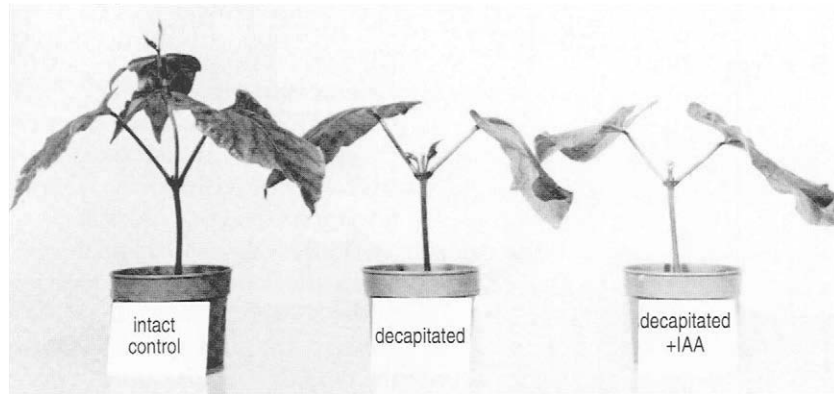


FIGURE 14-22 Apical dominance in runner bean (*Phaseolus*) plants. Sixteen-day-old plants were “decapitated” 5 days before the photograph was taken. Note that in the intact plant, lateral buds are inhibited; in the decapitated plant, the buds are growing out. In decapitated plants treated with IAA, buds fail to grow out. IAA (0.1%) in lanolin was applied to the cut stump. From Wareing and Phillips (1981).

decapitated, the whole cycle is repeated. Using this system, it has been shown that the mRNA of a ribosomal protein gene, *L27*, accumulates to high levels within 1 h after decapitation; the levels of the mRNA decline when the buds go quiescent again. mRNAs of two other genes, *PsDRM1* and *PsDRM2*, accumulate to high levels in quiescent buds, but decline sharply on resumption of growth following decapitation of the terminal bud.

3. IAA IS INVOLVED IN APICAL DOMINANCE

In a classic study, Thimann and Skoog (1933) showed that IAA applied in a lanolin paste to cut stumps of *Vicia faba* plants inhibited the growth of the lateral buds below. This experiment has been repeated by numerous other investigators using IAA as well as the synthetic auxin, 1-NAA, and there is no doubt that auxins can substitute for the shoot apex and enforce inhibition of lateral bud growth (Fig. 14-22). Such inhibition is better seen in plants with medium to strong apical dominance, much less so in plants with weak or no dominance. It should also be emphasized that such inhibition is caused only by auxins; application of other hormones, gibberellins, cytokinins, or abscisic acid, to the cut stump does not elicit the same response (Fig. 14-23).

Since IAA is synthesized in the shoot apex, especially young leaves, and shows a polar basipetal transport, it is postulated that it somehow causes the arrest

of lateral buds as it moves down. We will discuss this postulate later, but for the moment let us look at some additional evidence that IAA plays a role in lateral bud inhibition.

3.1. Inhibition of Polar Transport of IAA Causes Release of Lateral Buds from Arrest

As shown in Chapter 13, several chemicals, such as 2,3,5-triodobenzoic acid or 1-naphthylphthalamic

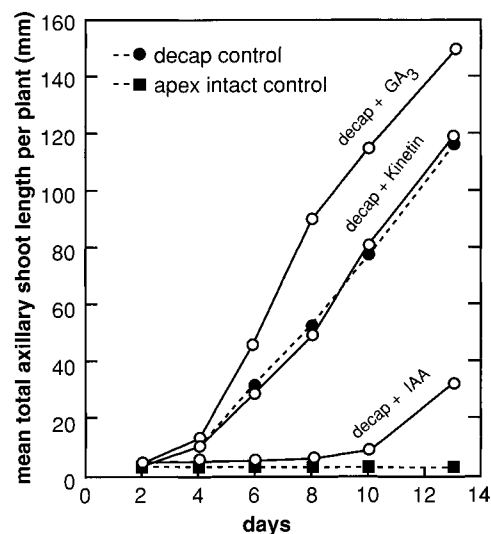


FIGURE 14-23 Growth of axillary buds in pea (*Pisum sativum*) plants following decapitation and treatment with different hormones. Hormones (1000 ppm) were applied in lanolin paste to decapitated stumps. From Wareing and Phillips (1981).

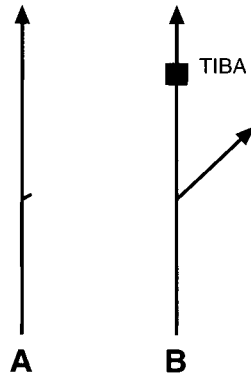


FIGURE 14-24 A schematic representation of apical dominance and its release after application of TIBA. The main axis and a single lateral bud are shown: (A) the lateral bud is arrested and (B) it has grown out.

acid, inhibit the basipetal transport of IAA. If one of these chemicals is applied in lanolin paste between the main apex and a lateral bud, the lateral is released from arrest (Fig. 14-24).

3.2. Alterations in IAA Levels in Transgenic Plants Affect Branching Patterns

Tobacco or petunia plants, which have been transformed genetically using the *iaaM* and *iaaH* genes from *Agrobacterium* under a constitutive promoter and overproduce IAA, show reduced lateral branching in transformed than in untransformed controls. The plants also show stunting and epinasty of leaves (Fig. 14-25A). In contrast, plants transformed with the *iaaL* gene from *Pseudomonas savastanoi*, which increases the conjugation of IAA to the amino acid lysine and, thus, leads to a decline in free IAA levels, show reduced apical dominance and enhanced branching (Fig. 14-25B).

3.3. How Does IAA Inhibit Lateral Bud Growth?

Unfortunately, the answer to this question is a mystery. While there is abundant evidence that IAA is involved in inhibition of lateral bud growth, there is still, after more than 60 years of research, no clear answer as to how it does so.

The very first theory proposed was that IAA flowing down the stem had supraoptimal concentrations near buds, concentrations that were inhibitory to growth. Subsequent work has shown, however, that this is not the case. No significant differences in IAA content in the nodes of buds that were arrested or growing after decapitation have been reported. *Ipomoea nil* is a flexible vine. If the stem tip is bent down and kept in an inverted position, the laterals are released from arrest, but there is no appreciable change in endogenous IAA levels at the nodes of the sprouting buds. There are other objections as well, not listed here, and the theory is no longer considered seriously.

A "nutritive" theory proposed that the main apex serves as a strong sink and draws nutrients, water, and photoassimilate away from the laterals, leaving the "poor" lateral buds more or less starved and, hence, unable to grow. A variation of this theory proposes that IAA in the main apex further enhances its "sink" capacity, relative to that of the laterals. This theory also suffers from serious objections. There is no convincing evidence that lateral buds are indeed deprived of nutrients, and provision of nutrients by exogenous means does not release them from arrest.

While there is still no consensus, most investigators now believe that although IAA plays a major role in apical dominance, it does so *via* interaction with other hormones, specifically cytokinins.

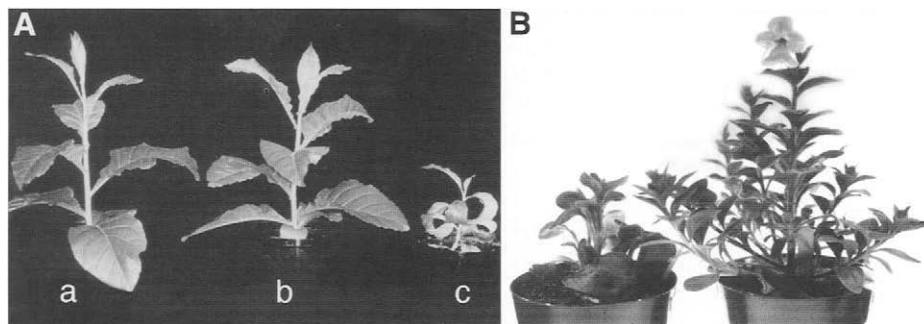


FIGURE 14-25 Wild-type and transformed tobacco and petunia plants. (A) Tobacco plants, wild type (a) and transformed with *iaaM* (b) and *iaaM iaaH* (c) genes from *Agrobacterium tumefaciens*. Transformation with a single *iaaM* or *iaaH* gene shows a relatively mild phenotype, whereas that with both genes shows a severe phenotype with stunting and epinasty of leaves. Courtesy of Folke Sitbon. (B) Petunia plants, wild type (left) and transformed by the *iaaL* gene (right) from *Pseudomonas savastanoi*. The transformed plant shows much more branching and is bushier than the untransformed control. Courtesy of Harry Klee.

4. CYTOKININS ARE INVOLVED IN RELEASE FROM APICAL DOMINANCE

Several lines of evidence show that cytokinins are involved in the release of lateral buds from apical dominance.

4.1. Transgenic Plants Overexpressing a Bacterial CK Synthesis Gene Show Enhanced Branching

The *ipt* gene from *Agrobacterium* encodes an isopentenyl transferase, which catalyzes the first step in CK biosynthesis. The coding sequence of this gene fused to a heat shock promoter and transfected to a higher plant, such as tobacco, causes much higher levels of endogenous CKs, zeatin riboside, and zeatin riboside-5'-monophosphate and much greater lateral branching than in the nontransformed controls (Fig. 14-26).

4.2. Exogenous Application of CKs to Buds Releases Them from Dominance

Sachs and Thimann (1967) demonstrated that application of CKs (kinetin) to arrested lateral buds of pea

plants caused them to be released from inhibition. Since then, many authors have reported similar results. Indeed, continuous treatment with CKs not only overcomes inhibition of lateral buds, but can even turn them into dominant organs. The effect is specific to CKs. Application of IAA or gibberellins to arrested buds has no effect, but once the release has been effected, growth of the lateral is enhanced by the application of IAA or GA (Fig. 14-27).

It should be emphasized that exogenous CK must be applied to the lateral bud itself. Application of CK alone or with IAA to the cut stump has no effect on release of the lateral bud from inhibition and may indeed reinforce inhibition (Fig. 14-28). In more recent years, these data have been confirmed by targeted expression of the *ipt* gene, linked to a light- or copper-inducible promoter. Such targeted expression in a single bud shows that increased CK levels in the bud itself are important, not globally in the plant.

Data from exogenous application of CK or localized enhancement in CK levels do not provide a clue as to what happens when a plant is decapitated or what effect polar transport of auxin may have on endogenous CKs. For this, endogenous CK concentrations must be determined.

4.3. Cytokinins Synthesized in Root and Transported via the Xylem Stream Cause Bud Release

Cytokinins are synthesized at various meristematic sites in the plant, including shoot apex, young leaves, cambial region, and, most importantly, root apices. CKs synthesized in the roots are transported upward into the shoot *via* the xylem stream. Using bean plants, it was shown that cutting the apex has a sudden and dramatic effect on the concentration of endogenous CKs in the xylem sap exuded out of the cut stump. The concentration of zeatin/zeatin riboside (Z/ZR) rose about 25-fold over the control 16 h after decapitation (Fig. 14-29); the rise in levels of isopentenyladenine/isopentenyladenosine was less but equally dramatic. Furthermore, application of the auxin 1-NAA to the stump at the time of the cut more or less completely repressed the rise in CK levels. The rise in CK levels in the exudate occurred before any significant elongation of the lateral buds, which suggested that CKs played a role in bud outgrowth. Similar results were obtained with pea plants, and it was proposed that cutting stimulates CK synthesis and export from the roots, which is normally kept under check by the polar basipetal migration of IAA (or, in this case, 1-NAA).



FIGURE 14-26 Tobacco plants, wild type (left) and transgenic plant (right) containing a heat-inducible *ipt* gene from *Agrobacterium*, were given daily 2-h heat treatments to induce expression. Relative to the heat-treated control plant, the transgenic plant exhibits substantially more growth of lateral shoots. Courtesy of Harry Klee.

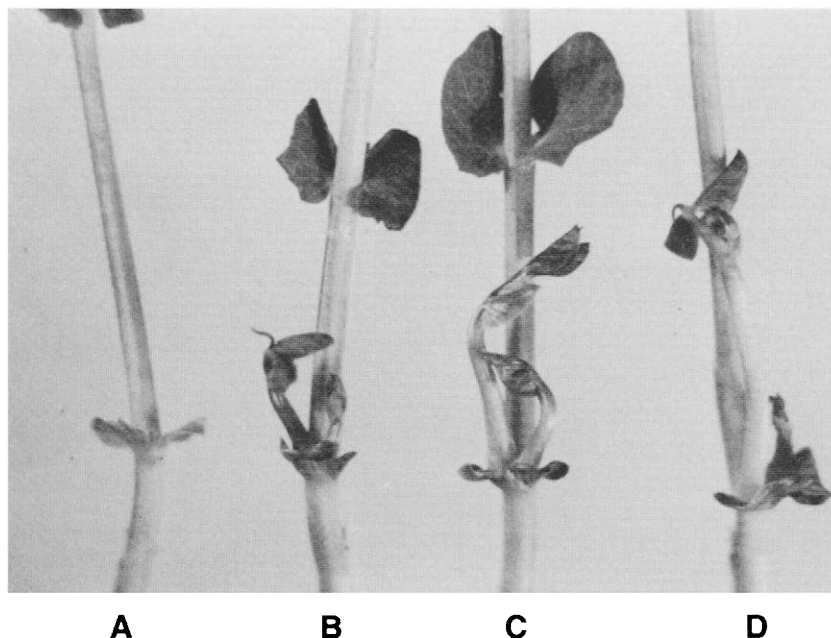


FIGURE 14-27 Release of axillary buds from correlative inhibition in intact pea plants. (A) Control, untreated bud; (B) bud treated with kinetin only; (C) bud treated with kinetin followed 3 days later by gibberellic acid (GA_3); and (D) bud treated with kinetin followed 3 days later by IAA. All hormones were applied directly to the bud. Concentrations used were 330 ppm kinetin, 100 ppm GA_3 , and 1000 ppm IAA. The photograph was taken 7 days after the beginning of treatment. From Sachs and Thimann (1967).

The picture that emerges therefore is that lateral buds are kept repressed by a certain ratio of endogenous IAA and CKs. If this ratio changes in favor of CKs, either by a cut at the apical end, which reduces IAA supply while increasing the endogenous supply of CKs from the root, or by direct CK application to the bud, the laterals are released from inhibition. The ratio

could be changed further, as mentioned in Section II,1, by overabundance of one hormone or the other. Thus, an abundance of IAA could reduce the free CK concentrations, or *vice versa*, an abundance of CKs could reduce the amounts of free IAA in the vicinity of buds. It needs to be emphasized that this ratio may be important in early steps of release from inhibition; later steps involving cell growth may well be promoted by IAA and/or GA.

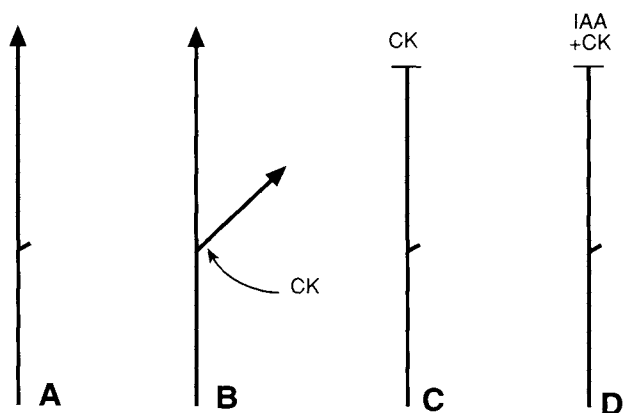


FIGURE 14-28 Diagrammatic representation of the positional effect of CK on the release of lateral buds from apical dominance: (A) control, (B) CK applied to the bud makes it grow, (C) CK alone, or (D) CK + IAA applied to the cut stump has no effect on the growth of the lateral.

5. THE DEVELOPMENTAL PROGRAM OF THE PLANT TAKES PRIORITY OVER REGULATION BY HORMONES

Studies with transgenic plants, or genetic mutants with altered branching patterns, have the advantage over decapitation studies in that they do not cause wounding or a sudden stoppage of basipetal auxin transport or a surge in cytokinin supply *via* the xylem sap. While generally supporting the hypothesis that an increased cytokinin to IAA ratio promotes lateral bud outgrowth, these studies have highlighted two important results: (i) regulation of lateral bud outgrowth by the IAA/CK ratio, in the first instance, is subject to the developmental program of the plant

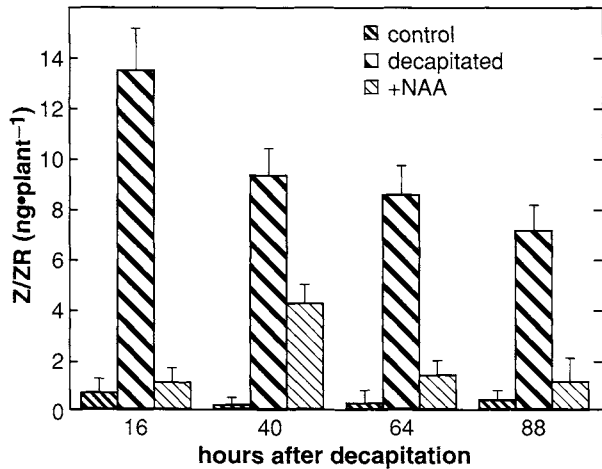


FIGURE 14-29 Amounts of zeatin/zeatin riboside (Z/ZR) in the xylem exudate of *Phaseolus vulgaris* at different times after decapitation. Plants were decapitated 1 cm above the node with two primary leaves at time 0. One set was left decapitated, while 1-NAA (0.15M in lanolin) was applied to the cut stump of the other set (+ NAA). Control plants were decapitated at the time of the sampling. Z/ZR levels were quantified using radioimmunoassay. From Bangerth (1994).

itself and (ii) the hypothesis may be too simple and other signals, in addition to IAA and CK, may be involved.

For instance, in plants transformed with the *ipt* gene, axillary buds show increased growth, but such growth usually occurs in upper nodes or in later stages of plant development when buds may be naturally released from inhibition. Similarly, plants transformed by the *iaaL* gene may show a significant drop in endogenous free IAA levels, but may not show enhanced branching until the time of flowering.

Mutants with altered branching patterns have been identified in several plants, including maize, tomato, *Arabidopsis*, petunia, and pea. Some of these mutants are pleiotropic in their effects, whereas others are more specific to branching.

The *tb1* (for teosinte branched 1) mutant of maize shows a much larger number of tillers (basal branches that grow out from the main stem) than normal maize (Fig. 14-30). The *Tb1* gene encodes a transcription factor, which acts as a repressor of axillary bud growth. Northern blots show that the gene is expressed in maize plants at nearly twice the level as in Teosinte and is maximally expressed in nodes where lateral branching is suppressed.

The *ls* (for lateral suppressor) mutant of some cultivars of tomato fails to produce axillary buds in leaf axils during vegetative growth (Fig. 14-31), although on transition to flowering, lateral buds are formed in the axils of the two youngest leaf primordia and grow normally. The mutant also lacks petals in flowers and shows reduced fertility. The defect has been traced to the shoot apical meristem and probably one of the peripheral tunica layers. The apices of homozygous *ls* plants contain significantly less cytokinin than the apical and axillary buds of the isogenic wild-type plants. However, because the defect is in lack of initiation of lateral buds, not their growth, increased cytokinin production by the transformation of plants by the *ipt* gene fails to induce branching in the *ls* mutant. The *Ls* gene encodes a protein that shares sequence similarity to the GRAS family of plant transcriptional regulators. Members of this family are believed to play diverse regulatory roles in tissue differentiation and in GA signaling (see Chapter 24). The function of the *Ls* protein in tomato is unknown.

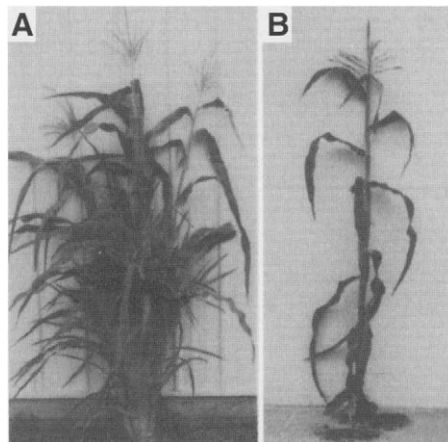


FIGURE 14-30 The teosinte branched mutant (A) and normal maize (B). The mutation causes a loss of apical dominance, resulting in a phenotype similar to that of Teosinte, the wild progenitor of maize. With permission from Doebley *et al.* (1997), ©1997 Macmillan Magazines Limited.

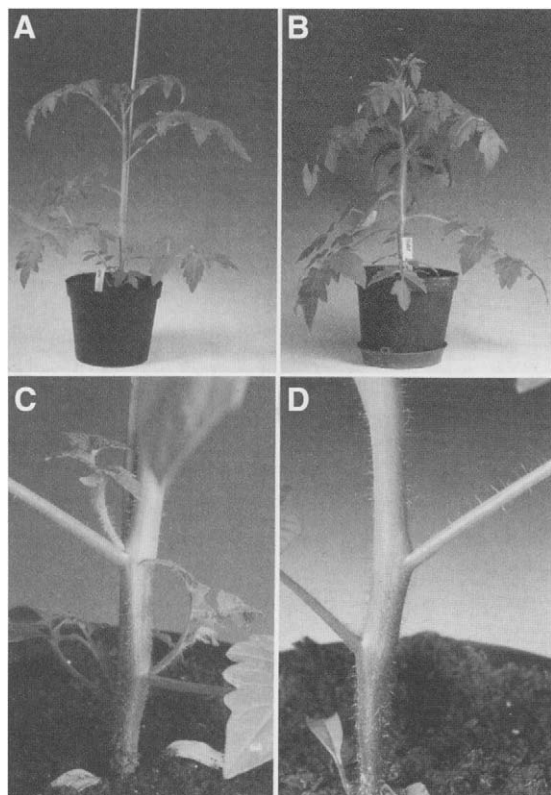


FIGURE 14-31 Comparison of phenotypes of wild type and *Is* mutant of tomato (*Lycopersicon esculentum* cv. Antimold B). The growth habit (A and B) and a close-up of leaf axils (C and D) are shown. From Schumacher *et al.* (1999)

Mutagenesis followed by screening for altered branching patterns has identified several mutants in petunia (e.g., *dad* for decreased apical dominance) and pea (e.g., *rms* for ramosus, Latin for having many branches). *rms* Mutants show enhanced branching, but no clear correlation exists between IAA content in tissues proximal to axillary buds and cytokinin levels in the xylem sap (Fig. 14-32). For instance, *rms1-1* shows high IAA content in stem segments and low CK content in xylem sap, but the *rms2-2* mutant has both high IAA and high CK contents. For some *rms* mutants, a graft-transmissible substance, which may come from the root but is not a major cytokinin, has been postulated as another endogenous factor regulating branching in pea. More recent data indicate that while auxin applied to decapitated wild type pea plants inhibits lateral bud outgrowth, this inhibition by auxin is severely curtailed in the *rms1* and *rms2* mutants. The basipetal transport of auxin is unaffected in the mutants, however. If *rms1* or *rms2* shoots are grafted on to wild type rootstocks, the auxin response is restored, which suggests that the product of the wild type *Rms1* and *Rms2* genes interacts with

exogenous auxin in inhibiting lateral bud growth following decapitation. Since the intact plants of *rms1* mutant are low in cytokinin content, these data suggest that auxin interacts with some other endogenous substance that is translocated long distance in the plant. The identity of the substance is unknown.

6. STRONG APICAL DOMINANCE IS CORRELATED WITH STRONG POLAR TRANSPORT OF IAA

Pea plants are a good experimental system because while dominance is seen in the main shoot, the other correlatively inhibited subordinate shoots can acquire dominance quickly when the principal shoot is decapitated. Two-branched pea plants have proven particularly useful in relating apical dominance to the polar transport of IAA. Such plants are produced by removing the epicotyl immediately above the cotyledonary node, 6–7 days after sowing. The two axillary buds at this node immediately grow out; after a few days, one of them continues to grow and becomes dominant, while the other becomes correlatively inhibited and subordinate (Fig. 14-33A). If the dominant shoot is decapitated, the inhibited subordinate shoot takes on the dominant role. Polar transport of [14 C]IAA has been studied in dominant and subordinate shoots in the intact two-branched plant, and it can be shown that such transport occurs at substantial velocities ($13\text{--}14\text{ mm} \cdot \text{h}^{-1}$) in the dominant shoot, whereas it occurs little or not at all in the subordinate shoots. If the dominant shoot is decapitated, polar transport starts in the hitherto subordinate shoot and soon reaches rates typical of a dominant shoot. Data for [14 C]IAA translocation in stem segments from dominant or subordinate shoots are shown in Fig. 14-33B. Figure 14-33C shows that in segments taken from subordinate shoots at varying intervals after decapitation of the dominant shoot, the capacity to transport radiolabel is gradually built up in 16–24 h.

Data just given clearly indicate that apical dominance correlates with the active polar transport of IAA. They also show that the capacity for polar transport of IAA is induced in a subordinate branch following decapitation of the hitherto dominant branch. It can be shown further that decapitation of the main shoot leads to a loss of the capacity for polar transport of IAA in the remaining parts of that shoot and, further, that such loss is prevented if IAA (or 1-NAA) is applied to the cut stump. Together these data indicate, though they do not prove, that, in young growing

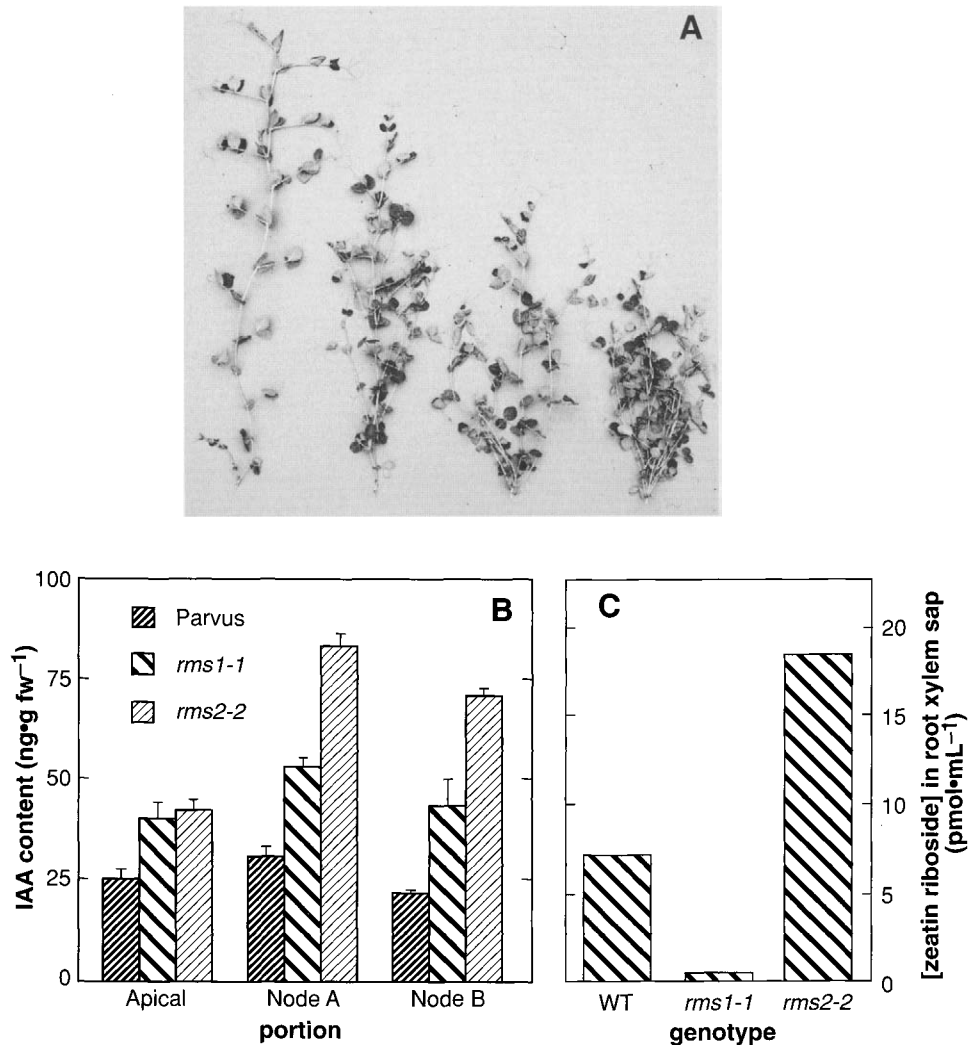


FIGURE 14-32 *rms* Mutants in pea and endogenous IAA and cytokinin levels in relation to the wild type. (A) Phenotypes (left to right) of 48-day-old cv Parvus (parent wild type), *rms1-1*, *rms2-2*, and *rms1-1 rms2-2* (double mutant) plants. (B) IAA content at three locations in cv Parvus, *rms1-1*, and *rms2-2* shoots. Apical, apical portion of the shoot above the oldest unexpanded leaf; Node A, the node at the highest expanded leaf; and Node B, the node below the highest expanded leaf. Nodal segments consisted of a 1-cm portion of the petiole and stem each side of the node. Data are means of three different pools of seven to eight plants. (C) Zeatin riboside ([9R]Z) concentration in the pooled root–xylem sap of cv Parvus, *rms1-1*, and *rms2-2* plants. $n = 8$ to 10. From Beveridge *et al.* (1997).

tissues, the efflux carriers, which are the basis for polar transport, are lost from the plasma membrane or “randomized” on decapitation and, conversely, on assumption of dominance, they are relocated in plasma membrane at the basal ends of transport-competent parenchyma cells.

6.1. Polar Transport of IAA in a Growing Lateral Bud

The acquisition of competence for polar transport requires an inducing signal. While the identity of

this signal is still unknown, it seems from data given earlier that the inducing substance is IAA itself. When lateral buds are released from arrest and are growing, they produce their own IAA, which moves down basipetally. As in the subordinate shoots of pea, which acquire competence to transport IAA basipetally, it is possible that channels for polar transport in the growing lateral buds are self-induced by IAA. This phenomenon has been referred to as “canalization” and finds further support in the differentiation of vascular tissues, discussed in Section VI below.

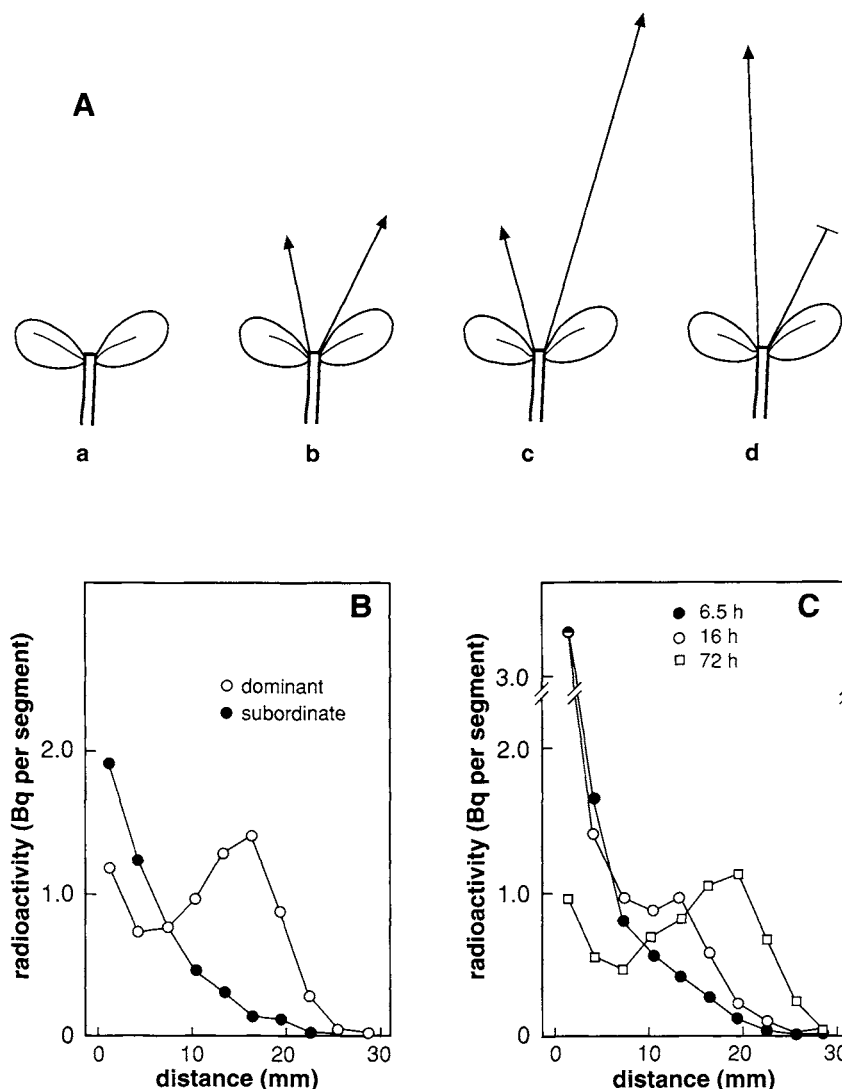


FIGURE 14-33 (A) Protocol for producing two-branched pea plants. (B and C) Polar transport of $[^{14}\text{C}]$ IAA in stem segments from dominant and subordinate shoots of two-branched pea plants. Thirty-millimeter segments were cut from dominant or subordinate shoots; the segments were pulse labeled with $[^{14}\text{C}]$ IAA ($3.4 \text{ mmol} \cdot \text{m}^{-3}$ in buffer, pH 5.0, + 1.5% sucrose) for 30 min and were then chased for 2 h with unlabeled IAA ($1 \text{ mmol} \cdot \text{m}^{-3}$). Means of three segments per treatment. (B) Transport of IAA in segments from dominant and subordinate shoots. In the dominant shoot much more radiolabel was translocated over longer distance than in the subordinate shoot. (C) Transport of IAA in segments from subordinate shoot taken at 6.5, 16, and 72 h after decapitation of the dominant shoot. From Morris and Johnson (1990).

7. ROLE OF OTHER HORMONES IN APICAL DOMINANCE

Other hormones do not seem to play a role in apical dominance or release of lateral buds from arrest. However, if the dominance over laterals is broken by CK application or by decapitation of the main apex, application of gibberellins promotes growth of the lateral buds. Exogenous ABA generally inhibits cell division and growth, and its application to the main growing

apex, like wounding or decapitation, releases the laterals from inhibition. A correlation is also suggested between ABA content of buds and their quiescent state, but precise quantitative data are lacking.

8. SECTION SUMMARY

Apical dominance is a complex phenomenon, which is determined genetically. Some plants show

strong apical dominance, others show moderate, and still others show weak dominance or none. In herbaceous plants that have been studied, apical dominance has been thought to be regulated by the relative concentrations of IAA and cytokinins at the bud site. The polar basipetal transport of IAA in the shoot and cytokinins transported upward in the xylem stream from roots play important roles in this phenomenon, but how the two hormones regulate bud quiescence or release from arrest is not clear. Once release has been affected, application of auxins as well as gibberellins promotes bud growth. Studies utilizing branching mutants and transgenic plants expressing altered levels of IAA or cytokinins generally indicate that the branching patterns, in the first analysis, are controlled by the genetic makeup and the developmental program of the plant. They also suggest that some factor other than cytokinin may be interacting with auxin in regulation of branching. A plant as an integrated whole is also subject to various environmental stimuli, such as photoperiod and proximity to neighbors, and resource allocation. There is a correlation between apical dominance of a shoot and a strong polar basipetal transport of IAA. Because apical dominance can be lost (by a dominant shoot on decapitation) or acquired (by a subordinate shoot), it suggests that the auxin efflux carriers, which are responsible for polar transport, can be lost from or randomized in the plasma membrane, and also selectively relocated at the basal ends of the plasma membrane of transport-competent cells. Moreover, the polar flow of IAA may itself provide the inducing signal for such relocation of efflux carriers.

SECTION VI. IAA AND DIFFERENTIATION OF VASCULAR TISSUES

In growing roots and shoots, procambial strands typically differentiate in an acropetal manner toward the root and shoot apices. Primary xylem elements and, for the most part, primary phloem elements also differentiate acropetally in the procambial strands (see Esau, 1957; Raven *et al.*, 1998). Some researchers have related the differentiation of primary vascular tissues to the presence of an inducing signal and suggested that IAA is such a signal. Differentiation of phloem tissues is usually difficult to follow; hence, most studies have utilized xylem differentiation. This section deals mainly with xylem differentiation, a process known as xylogenesis. It is shown first that IAA is required for xylogenesis and that IAA acts as

an inducing signal for setting up channels for its own polar transport a process known as "canalization," as well as a morphogenetic signal for the differentiation of xylem strands.

1. IAA IS INVOLVED IN XYLOGENESIS

Application of auxin to pith or cortical explants or callus tissue in culture induces parenchyma cells to differentiate as tracheary cells. For example, pith explants from Jerusalem artichoke (*Helianthus tuberosus*), tobacco (*Nicotiana tabacum*), *Coleus blumei*, and lettuce (*Lactuca sativa*) placed in culture medium with suitable concentrations of an auxin (IAA or NAA) show an induction of tracheary cells. These xylem cells, although they do not significantly elongate or expand radially, nonetheless deposit a secondary wall in defined patterns, lignify, and show autolysis. Similarly, cortical cells in pea (*Pisum sativum*) root segments are induced to divide and differentiate as tracheary cells if an auxin is supplied. A requirement for cytokinin has also been suggested, but its role in xylogenesis is less clear. Tissue explants are usually rapidly depleted of endogenous IAA, whereas, in many cases, they are able to synthesize their own cytokinins (see Section 4.3, Chapter 4); hence the effects of added auxin on xylogenesis are easier to demonstrate. Moreover, exogenous cytokinins have an effect only if auxin is present in the culture medium.

Tracheary element differentiation in the model system of *Zinnia* leaf mesophyll cells requires both an auxin (NAA, ~ 0.1 mg/L) and a cytokinin (usually BA, ~ 0.2 mg/L) in defined concentrations (Fig. 14-34). This requirement has been studied using stage-specific gene expression as molecular markers (see Fig. 4-15B,C in Chapter 4). The first stage, that of dedifferentiation, requires no hormone; it is brought about by wounding, but the continued expression of stage I-genes needs auxin and cytokinin. Both auxin and cytokinin are essential for the induction and continuation of the expression of stage II-related genes. The third stage seems to require auxin and cytokinin, but also has a requirement for brassinosteroids. Uniconazole, an inhibitor of P450-cytochrome-dependent monooxygenase, inhibits the synthesis of BRs; it also inhibits the expression of stage III-specific genes, an inhibition that is relieved by supply of brassinolide.

Callus is usually regarded as an unorganized mass of cells (see Section III). If a growing bud is grafted on callus tissue, groups of parenchyma cells below

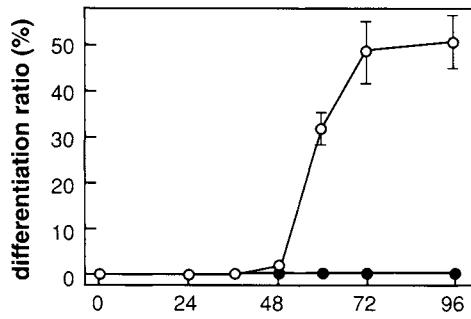


FIGURE 14-34 Tracheary element (TE) differentiation of *Zinnia* mesophyll cells cultured in the TE-inductive (o, NAA + BA) or a control medium (●, NAA). Each point represents the mean result from three samples ($n = 500$ in a sample), and vertical lines show standard deviations. With permission from Shinohara *et al.* (2000), © 2000 National Academy of Sciences, USA.

the graft differentiate as tracheary cells (Fig. 14-35). The same result can be obtained by placing an agar block with IAA on the callus tissue. The bud apparently produces IAA, which diffuses into the callus and induces the differentiation of xylem cells. Phloem cells (sieve elements) can also be induced in calli and usually occur at lower IAA concentrations than those needed for xylem differentiation.

Xylogenesis can also be induced in cortical cells of young seedlings if a flap of bark is lifted and hormones are applied to the inside of the lifted flap. In a study using young pine (*Pinus pinea*) seedlings, application of auxin alone led to differentiation of cortical parenchyma cells as xylem cells without much radial or longitudinal expansion. If auxin was supplemented with GA_3 , the differentiating tracheids showed significant elongation, but by itself GA_3 had no effect on xylogenesis.

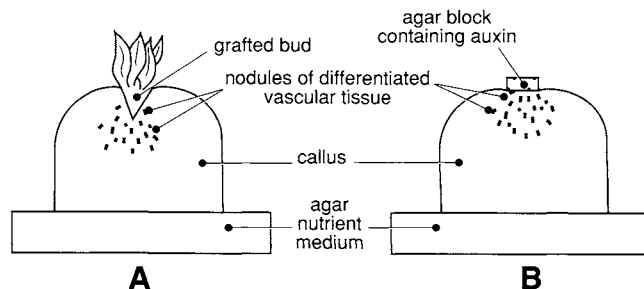


FIGURE 14-35 Differentiation of tracheary cells in callus culture of chicory by a grafted bud of the same species (A) and by exogenously applied IAA (B). From Wareing and Phillips (1981).

2. IAA ACTS AS A SIGNAL FOR BOTH CANALIZATION AND VASCULAR DIFFERENTIATION

The examples just given, while showing that IAA is necessary for xylogenesis, do not in every case involve polar transport of IAA. In intact plants, however, polar transport is responsible for the supply of IAA to the differentiating cells. If young leaves (and buds) are surgically removed from an otherwise intact plant, there is an immediate reduction in the number of differentiated xylem cells below the site of excision. If an auxin (IAA or NAA) is applied to the excision sites, there is no such reduction in xylem tissues.

One of the best examples of induction of xylem by polarly transported IAA is the regeneration of xylem cells following injury. If the vascular tissue in a stem or petiole is severed, the parenchyma cells in cortex and/or pith differentiate as xylem and phloem cells and establish vascular continuity. The regeneration of tracheary cells in the cut region follows the course predicted for IAA diffusing basipetally. The first differentiated tracheary cells occur just above the cut (where probably IAA accumulates), but most subsequent differentiation progresses basipetally. If the experimental plant is decapitated prior to severing the vascular tissues, the same pattern of regeneration of tracheary cells can be obtained by placing an exogenous source of auxin on the cut stump (Fig. 14-36).

Other experiments also indicate that xylem strands differentiate in a pattern that can be predicted from the path taken by polarly transported IAA. If blocks of storage parenchyma tissue from turnip roots are cut in a specific manner and auxin is applied as a single source, as in Fig. 14-37A, vascular strands differentiate in the parenchyma cells below and continue around the cut. When two sources are applied, one away from the cut (left) and the other above it (right), the pattern of differentiation indicates that the source on the left inhibits the flow of auxin from the source on the right (Fig. 14-37B). Most interestingly, if the source on the right is applied a few days before the source on the left, the source on the right is able to induce differentiation of its vascular strands (Fig. 14-37C). These data indicate that basipetal auxin flow induces vascular differentiation and also that the process of induction is gradual. For the pattern in Fig. 14-37C, an uninterrupted flow of IAA from the source on the right for almost 2 days was required.

Similar experiments using pith cylinders, bored from lettuce heads (for methodology, see Fig. 14-38), have revealed some interesting correlations. In these pith cylinders, differentiation of tracheary cells was

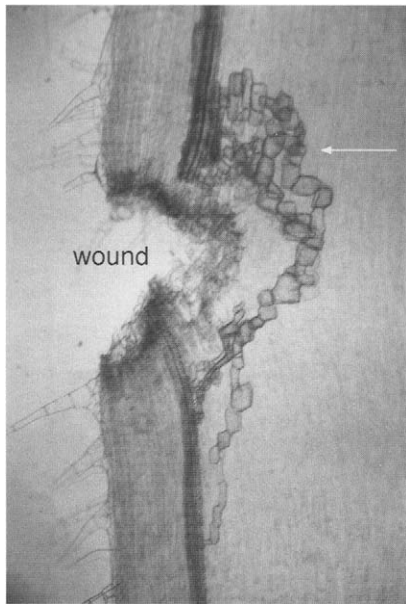


FIGURE 14-36 Differentiation of tracheary cells in cortex and pith after severing the vascular tissues in a *Cucumis sativus* plant. The plant was decapitated, the vascular tissues were severed by a deep cut, and an agar block with IAA was placed on the cut stump. Parenchyma cells in the cortex and pith differentiated as xylem cells (white arrow), reestablishing vascular continuity. Photograph taken 7 days after decapitation. From Aloni (1995).

restricted to the cut end where IAA was applied, presumably because polar IAA transport was limited, and movement was mostly by passive diffusion. Individual parenchyma cells differentiated as tracheary cells soon after IAA application (within 2 days), but the differentiation of tracheary cells in longitudinal files, i.e., xylem strands, took much longer (about 6 days), presumably after canalization had occurred. Also, in longitudinally bored cylinders, the strand length was longer if IAA was applied at the apical than at the basal end; it was shortest in cylinders that were bored transversely.

These observations on xylem regeneration in stems after wounding and vascular differentiation in storage parenchyma of turnip and in lettuce pith suggest that the diffusive movement of IAA delineates the files of cells that become competent for polar, basipetal transport, a phenomenon referred to as “canalization.” They supplement data from the acquisition of apical dominance by lateral buds and by subordinate shoots in pea after their release from dominance (see Section V,6). They are also in agreement with the distribution of PIN1 protein in embryo development as revealed by the *gnom* mutant and its role in establishment of shoot–root polarity (see Section III). Such canalization, or acquisition of competence for polar transport, involves synthesis and specific location of IAA efflux carriers on basal ends of cells and occurs over a few days. A rapid turnover of efflux carrier proteins in growing tissues (see Section 4.8, Chapter 13) would be an advantage in such a scheme.

Data also suggest that the diffusive flow of IAA serves as a morphogenetic signal for the differentiation of xylem cells in longitudinal files. Thus, IAA acts not only as an inducing signal for canalization, but also as a morphogenetic signal for differentiation of xylem strands. Such differentiation of primary xylem in an intact plant would appear to proceed acropetally in both shoots and roots following the pattern of apical growth and organ development. In a cut stem or petiole, however, where elongation growth has ceased, it would proceed basipetally.

While it is well established that IAA is involved in xylogenesis, the precise role(s) of IAA in tracheary cell differentiation remains unclear. As mentioned elsewhere, differentiation of these cells proceeds in several well-defined stages—radial expansion (in some cases also elongation), deposition of a secondary wall in precise patterns, lignification, followed by hydrolysis of nucleus, cytoplasmic contents, and, in vessel elements, parts of cell walls (see Chapter 2). IAA is known

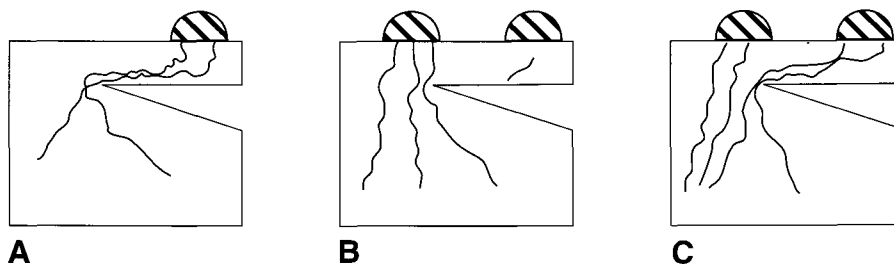


FIGURE 14-37 Evidence for canalization of vascular differentiation. Pieces of turnip (*Brassica napus*) storage root parenchyma were cut as shown, and IAA in agar blocks (hatched areas) was applied locally. Differentiation of vascular strands is shown by lines coming down from the top. From Sachs (1991) with kind permission from Kluwer.

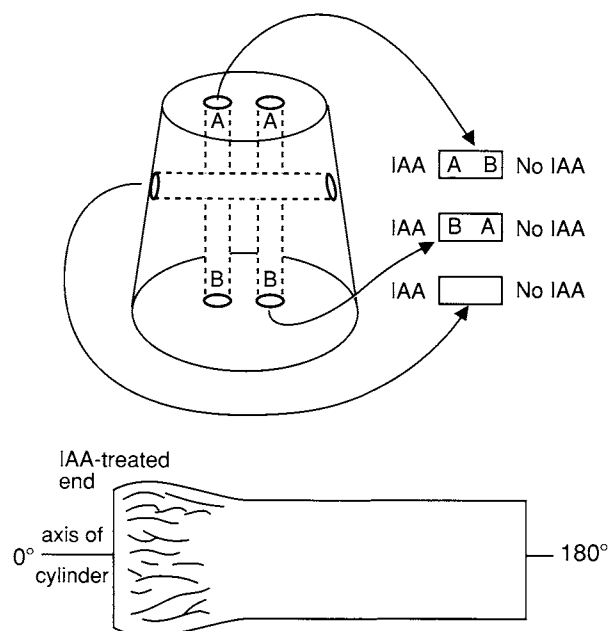


FIGURE 14-38 Differentiation of tracheary strands in pith cylinders from heads of lettuce (*Lactuca sativa*) var. "Lakes." (Top) Cylinders were bored from the head in three ways. They were sandwiched between agar blocks, and IAA was applied at one end as shown and incubated in a horizontal position to minimize the effect of gravity. Differentiation of tracheary cells or strands was checked microscopically at the end of the incubation period. In each case, tracheary cells differentiated close to the end where IAA was applied, and strand orientation was parallel to the long axis of the cylinders, shown as wavy lines (bottom). From Warren-Wilson *et al.* (1994).

to promote radial expansion in differentiating tracheary cells. Also, in suspension cultures, in which cells are inhibited from either cell division or cell differentiation by modifications of the culture medium, auxins are known to enhance cell expansion (see also Chapter 15). Some authors have suggested that the proton gradient responsible for the basipetal flow of IAA also regulates microtubular arrangements, which, in turn, are responsible for the oriented deposition of secondary walls. Auxin has been linked to lignification, but critical evidence showing such a link is lacking. Genes for many of the enzymes in the phenylpropanoid pathway for lignin biosynthesis are known, but there is no evidence that any of them is upregulated by IAA. IAA induces ethylene biosynthesis, and it is possible that ethylene is involved in xylogenesis, perhaps in the last phase of autolysis and cell death. Autolysis has also been related to the induction of serine and cysteine proteases, but a definite link between the induction of genes encoding these proteases and auxins has not been established. Finally, some data indicate that for the induction of tracheary cells in the *Zinnia* system,

auxin and cytokinins are only required for a short time, before any visible signs of differentiation; during the differentiation process, IAA is not needed in the medium.

Differentiation of phloem tissue, specifically sieve elements, has also been related to IAA. Most studies have centered around the regeneration of sieve elements after a cut. As in xylem differentiation, the precise role of IAA in phloem differentiation remains unclear, and it is possible that other substances, such as sugars, are also involved. For example, auxin/sucrose ratios have been implicated in both xylem and phloem differentiation; it has also been suggested that the differentiation of sieve elements occurs at a higher sucrose concentration than required for xylem differentiation.

3. IAA AND CAMBIAL ACTIVITY

Cambium is the tissue responsible for the production of secondary xylem (wood) and secondary phloem. Many aspects of cambial activity, including reactivation, xylem production, and formation of compression wood, have been related to the polar flow of IAA. These topics are discussed in the following section.

3.1. IAA Is an Important Factor in Reactivation of Cambium in Spring

In temperate climates, vascular cambium becomes dormant in the fall and resumes meristematic activity in the spring. It is commonly assumed that IAA is involved in cambial reactivation, i.e., induction of cell division activity. It has also been assumed that cambial activity proceeds from the top of the trunk to the base, a view that may be derived from the fact that IAA is produced in flushing apical and lateral buds and young shoots and flows basipetally. There is some evidence for a basipetal progression of cambial activation in diffuse porous woods based on bioassays. However, studies on cambia of conifers as well as diffuse- and ring-porous dicot woods, while demonstrating that IAA is required for cell divisions in the cambial zone, do not support the assumption that cambial activation proceeds basipetally in the main trunk. Such basipetal progression is seen only in young parts of a tree, usually the first year's growth; the rest of the trunk is reactivated more or less simultaneously.

Measurements of endogenous IAA in tree trunks at different heights using modern methods of analysis and quantitation are very few. They are also difficult because sampling pieces of bark, cambium, and wood from tree trunks takes time and quick freezing of rela-

tively large samples in liquid nitrogen or isopentane still does not stop the mobility of small molecules and ions instantaneously. Nonetheless, studies have been made and indicate that the situation is more complex than previously realized. A vertical gradient in IAA concentration is seen mostly in young stems and branches and in trees that are growing vigorously. The gradient is not so clear and may even be nonexistent in older stems or in slow-growing trees. Moreover, not all IAA moving down basipetally comes from the shoot apex. Feeding ^{13}C -labeled IAA to a decapitated pine shoot showed isotopic dilution down the trunk, which suggested that at least some IAA in the trunk is synthesized locally at lower levels. Finally, dormant cambium also has significant amounts of IAA, which could be mobilized in spring.

The site of polar transport of IAA in tree trunks is thought to be the cambial zone. It has been mentioned before that it is possible to measure very small quantities of hormones in tissue sections or small samples (see Chapter 5). In several papers, IAA concentrations were monitored in individual tangential sections of a pine stem and data were integrated to give a profile of IAA concentrations in the cambial zone and differentiating and mature secondary xylem and phloem cells on either side (Fig. 14-39). Data show that the highest concentrations of IAA occur in the cambial zone and fall off in a gradient on either side in the differentiating secondary xylem and secondary phloem, with fully mature tissues showing very little IAA.

It would be expected that the IAA concentration in the cambial zone at any one location in the trunk would be higher in spring/summer when cambium is actively producing xylem and phloem than in winter when it is dormant. However, the summer and winter samples did not show much seasonal fluctuation, although there was a broadening of the IAA gradient in spring/summer and a narrowing of the gradient in winter (Fig. 14-39B). The presence of IAA in the dormant cambium suggests, by inference, that the cessation of cambial activity in late summer–early fall is not controlled by IAA, a suggestion that is supported by feeding experiments where IAA supplied to shoots does not prevent the cambium from becoming dormant. Environmental factors, such as temperature and shortening daylength, seem to be involved in the induction of cambial dormancy. Although the concentration of IAA did not show much seasonal variation, the active cambium contained a greater amount of IAA than the dormant cambium, which indicates that higher amounts of IAA are produced and utilized, i.e., there is a higher flux of IAA in the cambial zone in the summer months. The observation that the IAA content in differentiating xylem and phloem tissues was about

the same is difficult to explain because higher IAA concentrations are known to promote xylem differentiation (see below). It could be that other factors besides IAA, such as sugars and gibberellins, may also control the developmental fate of cambial derivatives.

3.2. IAA and Gibberellins May Regulate the Production of Secondary Xylem and Phloem

The activation of cambium and the differentiation of secondary xylem and phloem can also be studied in cut stem segments of annuals with secondary growth (e.g.,

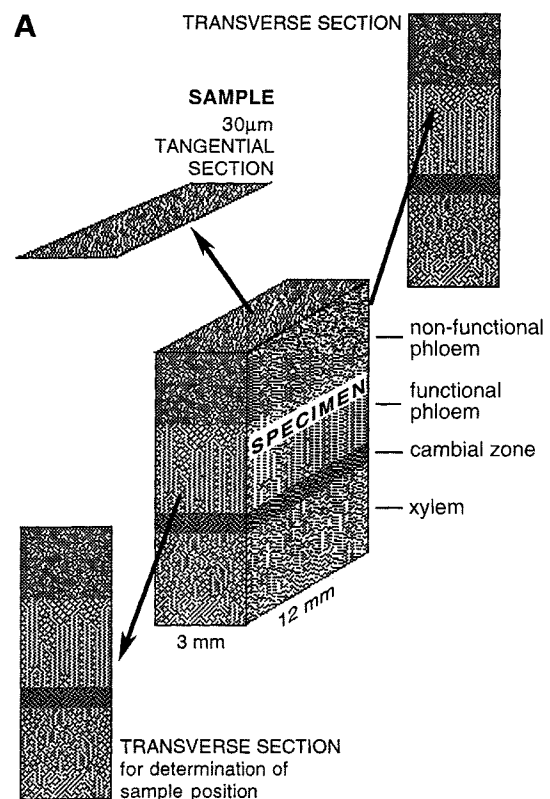


FIGURE 14-39 Schematic drawing of the specimen block and radial distribution of IAA in the cambial zone and secondary tissues of pine (*Pinus sylvestris*). (A) Tangential longitudinal sections (30 μm in thickness, using a cryomicrotome at -20°C) were obtained starting from the outer phloem and into the xylem tissue. IAA content was measured in each section (sample) using a modified GC-MS procedure. Transverse sections at ends were used for the determination of sample position. (B) Radial distribution of IAA in two representative trees; one sampled in late June at the height of cambial activity and the other sampled during dormancy in mid-January. Each column represents the 30-μm tangential section. Endogenous IAA content per cm^2 section is indicated with black squares. NFP, nonfunctional phloem; FP, functional phloem; CZ, cambial zone; ET and DT, expanding and differentiating tracheids; MT, mature tracheids. The average number of radial file cells in each developmental zone is given on the right. With permission from Uggle *et al.* (1996), ©1996 National Academy of Sciences, USA.

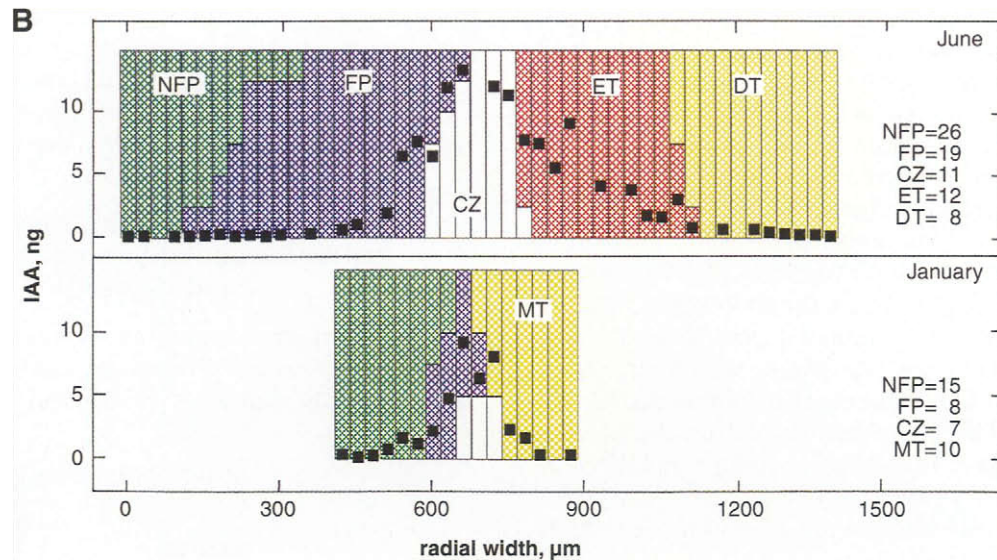


FIGURE 14-39 (continued)

tomato, bean) or trees, such as birch (*Betula*), poplar (*Populus*), and black locust (*Robinia*). If tree samples are taken in the fall, it is essential to give them a cold treatment at 2–5°C for several weeks to break their winter dormancy. These samples, given appropriate IAA and/or gibberellin, treatment, show that while IAA and GA both promote cambial reactivation, IAA favors xylem and GA favors phloem differentiation (Fig. 14-40).

3.3. Auxin Is Involved in Compression Wood Formation

When trees lean to a side or produce branches that are at an angle to the main trunk, reaction wood is

formed by asymmetric growth on the lower vs the upper side. Such asymmetric growth results from the differential activity of cambial initials or their immediate derivatives, such that more xylem cells are produced on one side than on the other. Conifers and hardwoods differ in the nature of reaction wood. In hardwoods, the reaction wood is formed on the upper side and, although rich in cellulose, is much less lignified (tension wood), whereas in conifers, it is formed on the lower side and is heavily lignified (compression wood). The formation of compression wood in conifers can be induced by the application of a supraoptimal concentration of auxin on one side, or even all the way around.

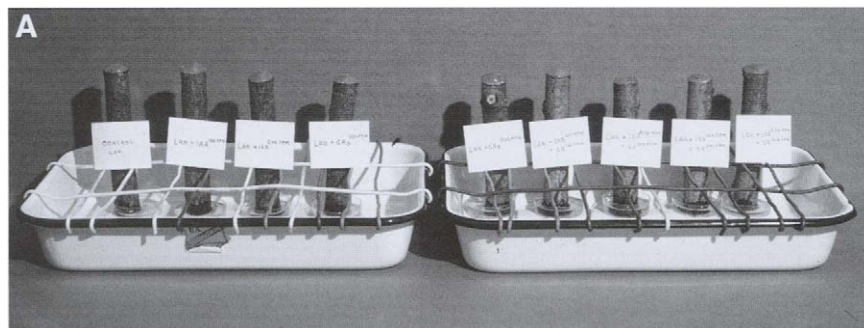
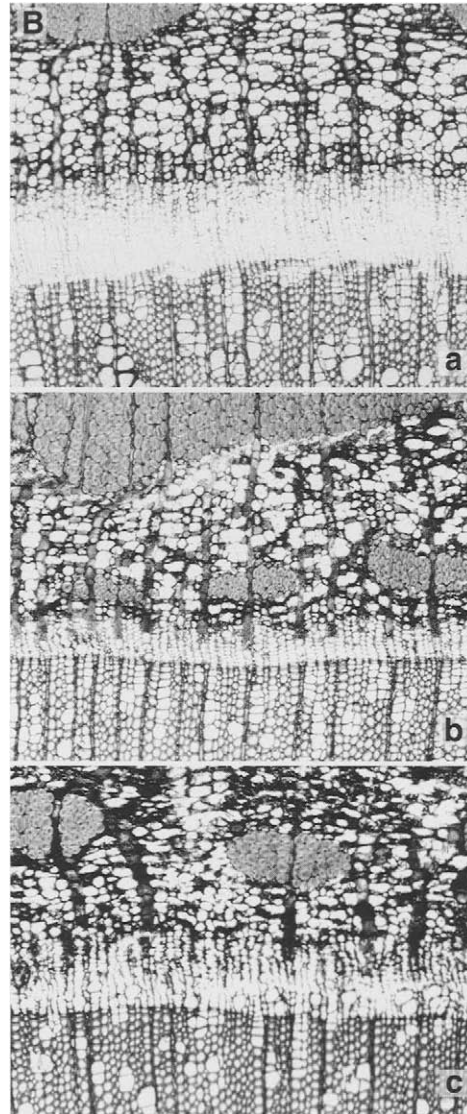


FIGURE 14-40 Activation of cambium and differentiation of xylem and phloem in stem segments of *Robinia pseudoacacia* (black locust). (A) Experimental protocol. Branches were sampled in the fall and placed in a cold room for 4–6 weeks to break winter dormancy. They were cut in 10- to 12-cm lengths and placed, morphological side up, in small petri plates with some water, and different concentrations of GA₃ and/or IAA in lanolin were applied to the top end. After 4 weeks, sections were obtained from the segments, using a sliding microtome, and stained for xylem and phloem. (B) Cross sections of stem segments: (a) 100 μM GA₃, (b) lanolin only, and (c) 100 μM IAA. Note that application of both IAA and GA promotes cambial activation, but IAA promotes xylem differentiation and GA promotes phloem differentiation. From Lai (1974).



Auxin concentration is not the only factor regulating xylogenesis. As mentioned earlier in Sections VI,1 and VI,2, ethylene and cytokinins may also be involved, although their roles are not clear. In one study, auxin polar transport inhibitors (NPA or morphactin) applied as a ring around the stem completely inhibited polar auxin transport and new xylem formation below the site of application, while inducing compression wood formation all around the stem above the application site. However, measurements of endogenous IAA did not show any significant accumulation of IAA, or IAA conjugates or breakdown products, above the site of application. High concentrations of IAA induce ethylene production, and the above results could have been due to ethylene.

4. SECTION SUMMARY

There is abundant evidence from the application of auxin to defoliated or disbudded stems, cortical or pith explants, callus tissues in culture, and parenchyma in stem flaps that auxins promote xylogenesis. If the vascular tissues are severed in a stem or petiole, basipetal transport of IAA induces the regeneration of xylem and phloem tissues in parenchyma cells of pith and/or cortex to reestablish vascular continuity. Similarly, in blocks of parenchyma cells from storage tissue or pith cylinders, the basipetal flow of IAA induces the differentiation of vascular strands. It appears from these and other experiments that IAA acts as an inducing signal to set up its own channels for polar trans-

port, a phenomenon known as canalization. Second, if conditions are right, IAA also acts as a morphogenetic signal for the induction of xylem strands. In conifers and hardwoods growing in temperate climates, IAA is a major factor responsible for the activation of vascular cambium in the spring. In young shoots, cambial activity depends on the *de novo* synthesis of IAA in expanding buds, but in old trunks, sufficient IAA may already be present in the cambial zone for the initiation of cell division activity. To maintain cell divisions and xylem and phloem production, the cambium must be supplied with IAA by polar transport and/or localized synthesis. The production of secondary xylem, like that of primary xylem, is favored by high concentrations of IAA or synthetic auxin. Some data suggest that increased gibberellin concentrations favor phloem differentiation.

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SECTION I. AUXIN AND CYTOKININ HOMEOSTASIS

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For auxin or cytokinin-overproducing mutants, see Chapters 6 and 8; for transgenic plants, see Appendix 2

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15

Hormonal Regulation of Cell Division and Cell Growth

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1. INTRODUCTION

Cell division and cell growth are two fundamental processes that regulate the development and morphogenesis of plant organs (e.g., roots, stems, leaves). Frequently, cell division and cell enlargement go together, but in some cases, growth may be due mostly to cell enlargement. For instance, the auxin-induced growth of cut coleoptile segments has no cell division component. The gibberellin (GA)-induced growth of lettuce hypocotyls is primarily due to cell elongation, but there is some contribution from cell division,

which is confined to the apical part (~0.5–1.0 mm) of the hypocotyl. Plant hormones play important roles in both cell division and cell enlargement. This chapter deals with the question of how hormones regulate cell division and then how they regulate cell enlargement.

SECTION I. CELL DIVISION

1. HORMONES REGULATE CELL DIVISIONS IN INTACT PLANTS, CUT SEGMENTS, AND CALLUS AND CELL CULTURES

That hormones regulate cell division in plants has been recognized since the late 1950s. Three hormones in particular, GAs, indoleacetic acid (IAA), and cytokinins (CKs), are known to induce cell division, whereas ABA inhibits it. The role of brassinosteroids (BRs) in cell division is still uncertain.

1.1. Cell Divisions in Whole Plants or Organs

As explained in Chapter 1, root and shoot apical meristems, intercalary meristems, and lateral meristems, such as vascular cambium, are sites of meristematic activity, subject to seasonal and/or developmental regulation. In addition, sporadic, localized cell divisions occur in various locations in the plant body. These divisions during normal growth are probably regulated by endogenous hormones, although an unequivocal demonstration of such regulation in whole plants is not easy. There are a few situations, however, such as the auxin-induced formation of lateral or adventitious roots and cytokinin-induced bud outgrowth (see Chapter 14), that provide the possibility for an experimental verification of hormonal effects on cell division. One such experiment on lateral root induction is described in Section I,2.1. Two other excellent examples of GA-regulated cell divisions follow.

1.1.1 GA-Induced Cell Divisions in Rosette Plants

One of the classic studies showing GA-induced cell divisions comes from the work of the late Anton Lang and associates at MSU-DOE in East Lansing, MI. These authors studied the phenomenon of “bolting” in rosette plants, such as *Hyoscyamus niger* (henbane) and

Samolus parviflorus. Bolting involves very rapid growth of the flowering axis by both cell division and cell elongation and is known to be regulated by GAs. Every 24 h they injected GA into the bases of rosette leaves proximal to shoot apices. Apices were sectioned, and mitotic figures were mapped and enumerated 24 to 72 h after the start of treatment. Figure 15-1 shows a correlation between GA and mitotic figures in henbane, and it is clear that GA treatment had induced cell divisions and that most divisions were in the sub-apical zone.

1.1.2. Intercalary Meristem in Deepwater Rice

Floating or deepwater rice (*Oryza sativa*) is an important crop in tropical countries of southeast Asia. It is grown in low-lying areas and is subject to periodical flooding during the monsoons when waters may rise several meters in a few days. These rice cultivars have an extraordinary capacity for growth, to literally keep their “heads” above rising waters and escape anoxia. In the field, they are known to grow up to 25 cm per day! This growth, seen mainly in the youngest internode, involves both cell division and cell elongation and is a classic example of a defined response, i.e., growth, being regulated by several hormones. Flooding causes a reduced partial pressure of O₂ in submerged tissues, which causes enhanced synthesis of ethylene. An increased concentration of ethylene results in a reduction in endogenous ABA content, which, in turn, increases the sensitivity of the tissue to endogenous GA. GA is the ultimate hormone that stimulates both cell division in the intercalary meristem and elongation of its derivatives. This remarkable chain of events was worked out by Hans Kende and associates at MSU-DOE in a series of papers spanning more than a decade. In the laboratory, detached stem sections, consisting of the youngest internode and leaf sheaths and developing leaves, can be made to grow if placed in a container of water, simulating flood conditions, or, alternatively, if treated with exogenous GA (Fig. 15-2).

1.2. Cell Proliferation in Suspension Culture

As shown in Chapter 14, cell proliferation, as well as shoot bud and root induction, in callus explants is regulated by an exogenous supply of auxin or auxin and a cytokinin. Cells derived from calli, or plant organs such as leaf mesophyll, in suspension culture

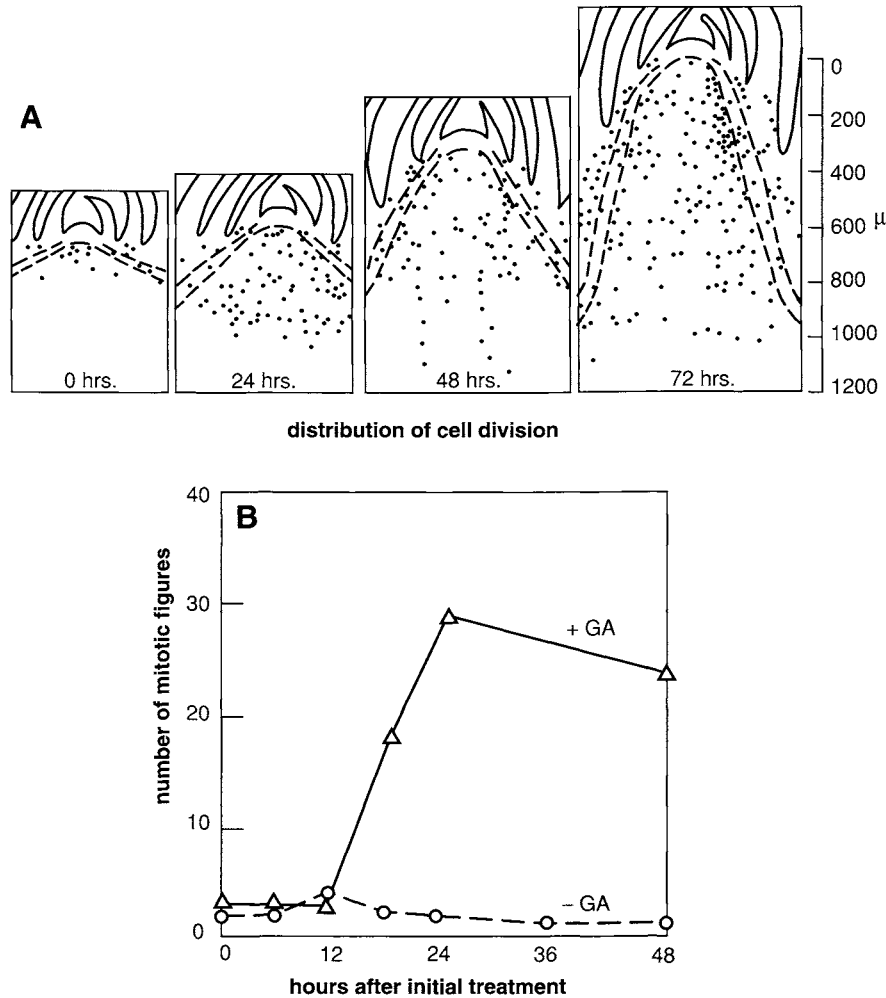


FIGURE 15-1 GA induction of cell division in the shoot apex of *Hyoscyamus niger*. (A) Longitudinal sections of the shoot apex at different times following application of GA₃. Each dot represents one mitotic figure in a 64- μ m-thick section. Divisions are mostly in transverse plane, thus resulting in elongation or "bolting" of the stem. (B) Plots of numbers of mitoses in the subapical zone with and without GA₃. From Sachs *et al.* (1959).

provide an excellent material to study the hormonal regulation of cell division.

2. HORMONES REGULATE EVENTS IN CELL CYCLE

How do auxins, CKs, and GAs induce cell division in whole plants/organs, in calli, or in suspension cultures? As reviewed in Chapter 2, the cell cycle in eukaryotes has checkpoints at the G1/S transition and at the G2/M transition, and there are specific G1 and mitotic cyclins that partner with their cognate cyclin-dependent kinases (CDKs) to phosphorylate

substrate proteins that take the cells over the two checkpoints. Genes and cDNAs of G1 and mitotic cyclins and several CDKs have been cloned from plants (see Chapter 2). The availability of these molecular probes has opened a new way to examine the status of the cell cycle in plants and particularly to study the effects of individual hormones on the induction (or loss) of expression of a specific cyclin or a CDK. These studies are still relatively few, but their potential in unraveling the hormonal regulation of cell division is great. Before reviewing them, it is important to understand some of the methodologies used in these studies. These methodologies are summarized in Box 15-1.

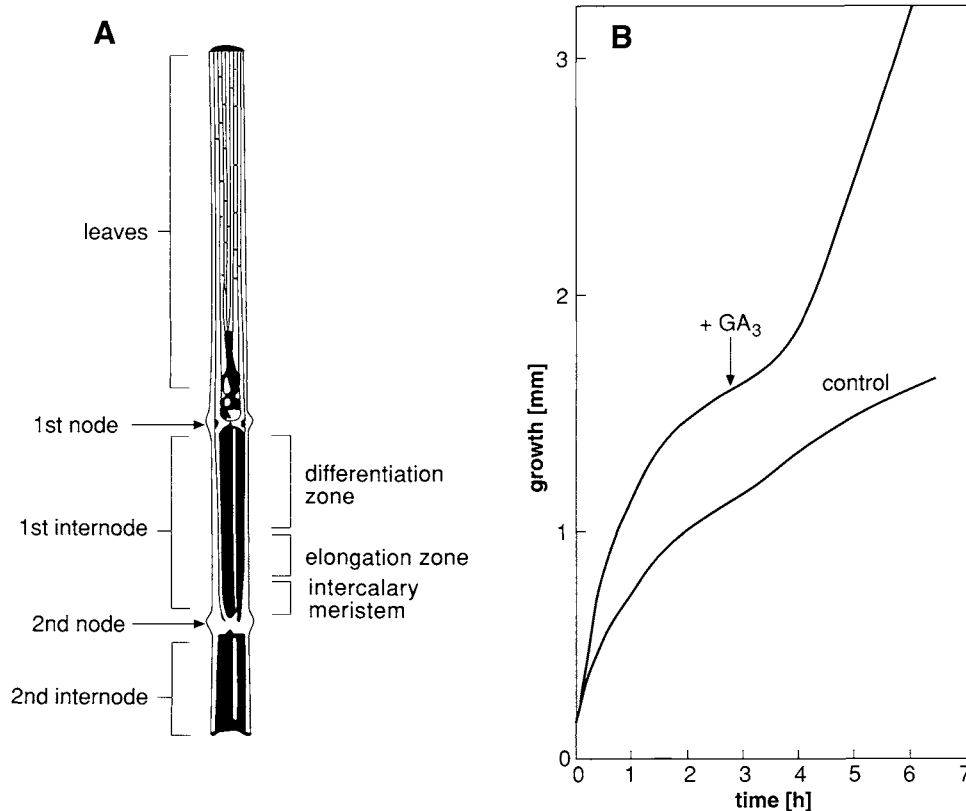


FIGURE 15-2 GA-induced growth of young internodes of deepwater rice (*Oryza sativa*). (A) Longitudinal median section through a 20-cm-long stem section. The youngest, or first, internode occurs between the first (highest) node and the second node. In this internode, locations of the intercalary meristem, elongation zone, and differentiation zone are indicated. The section above the first node consists of leaf sheaths and developing leaves. (B) Continuous recording of internodal elongation in the control (lower curve) and GA₃-treated section. Growth was recorded using an angular transducer. GA₃ (50 μ M) was applied at the time indicated by the arrow. While the pattern of the growth response was similar in different stem sections, the magnitude of the response varied between sections, as seen here during the first 3 h of growth. From Sauter and Kende (1992).

BOX 15-1 DETERMINATION OF SPECIFIC STAGES IN THE CELL CYCLE

SEVERAL METHODS ARE USED, often in combination, to study specific stages in cell cycle and their regulation. Both callus cultures and whole plants/organs have been used to study the hormonal effects.

Synchronization of cells in suspension cultures: Suspension cell cultures may be derived from calli or from plant tissues such as leaf mesophyll. Cells from calli or freshly harvested leaf tissue have cell walls, which are enzymatically digested to yield protoplasts. The protoplasts are placed in liquid nutrient media with a carbon source, sucrose, and an auxin [2,4-dichlorophenoxyacetic acid (2,4-D) or naphthalene acetic acid (1-NAA)] and a cytokinin (usually BA). In 2–3 days, depending on material, protoplasts form nascent cell walls and begin to divide (Fig. 15-3A). In cell division literature, the term “protoplast” is used to denote individual cells with regenerated walls. The cell population increases rapidly at first and then slows down as the culture reaches the stationary phase (Fig. 15-3B). To trigger the cells to divide again, a batch of cells is inoculated into fresh medium with sucrose plus auxin and cytokinin.

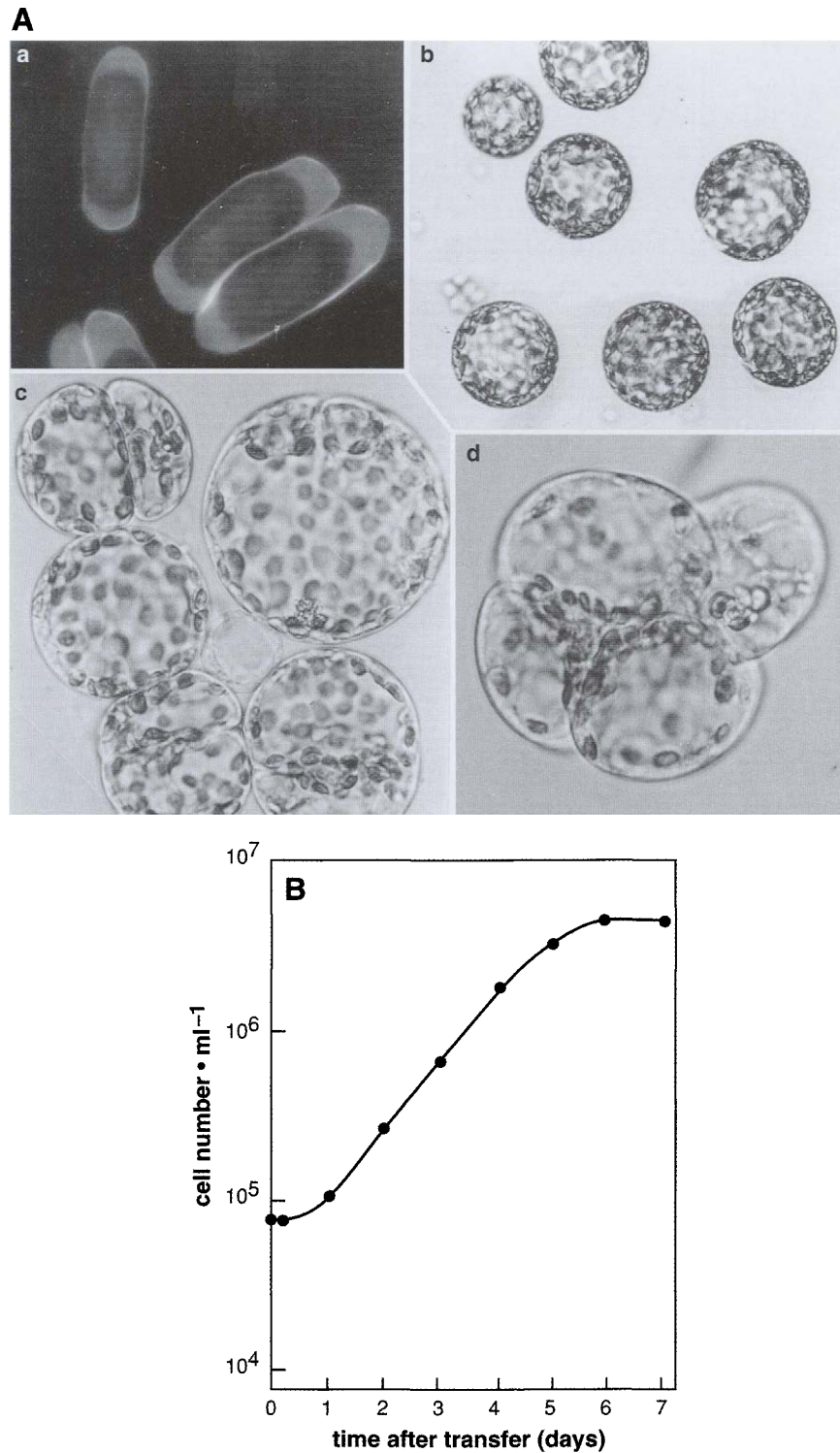


FIGURE 15-3 (A) Protoplasts from leaf mesophyll cells of tobacco. (a) Freshly isolated palisade parenchyma cells stained with Calcofluor White ST, a specific fluorescent stain for cellulose. The cells are plasmolyzed because they are suspended in 0.7 M mannitol. $\times 340$. (b) Freshly isolated protoplasts. $\times 340$. Cultured protoplasts undergoing first (c) and second (d) cell division. $\times 525$. Courtesy of Toshiyuki Nagata, University of Tokyo. (B) Growth curve of tobacco Bright Yellow 2 (BY-2) cells in culture. Growth was monitored by counting the cell number per 1 ml at approximately 1-day intervals. The stationary phase is reached after approximately 6 days. From Nagata *et al.* (1992).

Cells in suspension cultures occur in different stages of the cell cycle. To study the effect of hormones on cell division, it is useful to get them to synchronize their divisions, such that most cells are in the same stage of the cell cycle. Such synchronization is obtained by taking cells in the stationary phase and inoculating them in fresh medium with chemicals that block cells at a particular stage. Aphidicolin, a drug purified from culture filtrates of a fungus *Cephalosporium aphidicola*, is a potent inhibitor of DNA polymerase. Cells treated with aphidicolin for 24 h are arrested mostly in G1 and S phases. When washed and placed in fresh medium, they enter mitosis with considerable synchrony. Such synchrony is usually maintained over one or two cell cycles—the period used for study—but eventually is lost. Hydroxyurea (HU) inhibits the enzyme ribonucleotide reductase; it also blocks cells in G1 and S phases, but is less effective than aphidicolin. To improve synchronization, a second chemical that blocks mitosis (e.g., oryzalin, propyzamide, or colchicine, which depolymerize microtubules and arrest cells in metaphase) may be used (see Fig. 15-4). Using aphidicolin and propyzamide in succession with washes in between, the mitotic index can be raised to 40–50%, although it has been reported to be as high as 80%. Biochemical events of growth in these twice-blocked cells may not be in the same balanced state, however, as in undisturbed cells.

Cell lines from different plants differ in the ease with which they can be synchronized. Cell lines from tobacco (*Nicotiana tabacum*) cv Bright Yellow 2 (BY-2) under appropriate nutrient conditions are highly synchronizable and give vigorous growth. They are used for many cell and molecular biological studies on cell cycle. Other lines include those from the Madagascar periwinkle (*Catharanthus roseus*). As mentioned earlier, mesophyll cells from fully differentiated leaves of many plants are arrested in G1 (e.g., *Petunia hybrida*, *Asparagus officinalis*). A high proportion of freshly isolated protoplasts from these tissues shows a 2C DNA content and can be used directly without a need for synchronization. Here, too, synchrony is lost over time.

Synchronized cells or whole tissues supplied [^3H]thymidine incorporate it into the new DNA synthesized during the S phase. The incorporation of radiolabel in nuclei is a signal that cells are progressing from G1 to S phase. Histones are synthesized during the S phase as well, and gene-specific probes for histone H4 are used to confirm the transition to S phase (Fig. 15-4).

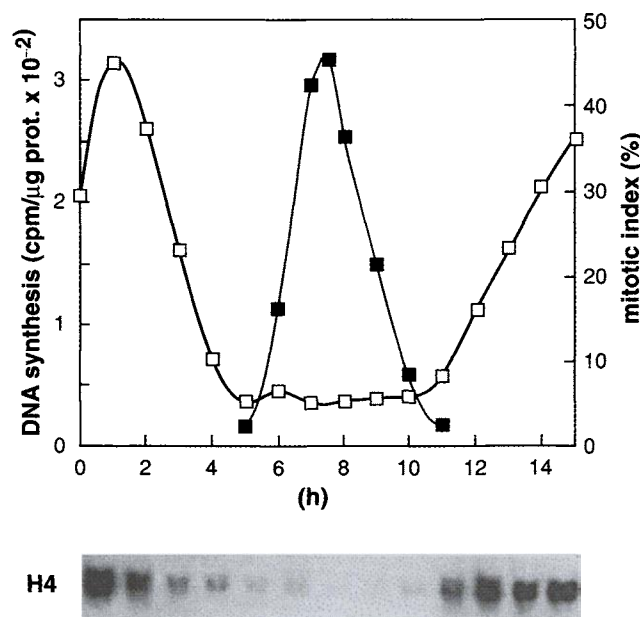


FIGURE 15-4 Synchronization of tobacco BY-2 cells. Two blocks, one inhibiting the S phase and the other mitosis, were used sequentially (with in-between washes) to obtain highly synchronized cells. DNA synthesis was determined by pulse labeling with [^3H]thymidine. The mitotic index was determined by staining with $10\ \mu\text{g}\cdot\text{ml}^{-1}$ DAPI (4,6-diamidodino-2-phenyl indole). Histone H4 was monitored by Northern blots using cDNA of the *Arabidopsis* H4A748 gene. Data for one complete cell cycle are shown. [^3H]-Thymidine incorporation (□ squares), mitotic index (■). Transcript levels of histone H4 are shown below. Modified with permission from Espunya *et al.* (1999), ©1999 Blackwell Science Ltd.

Determination whether cells are in G1 or in G2: There are various methods for measuring the DNA content of nuclei. One of the common methods is by flow cytometry. In flow cytometry, isolated nuclei that have been stained with a DNA-specific fluorescent stain, such as propidium iodide, are made to flow through a capillary. Fluorochrome bound to the nuclei is excited by light of a suitable wavelength and emits fluorescence in proportion to the DNA content. The fluorescence is measured, plotted, and displayed to indicate the proportions of nuclei that are in 2C (G1), 4C (G2), or higher multiples (Fig. 15-5).

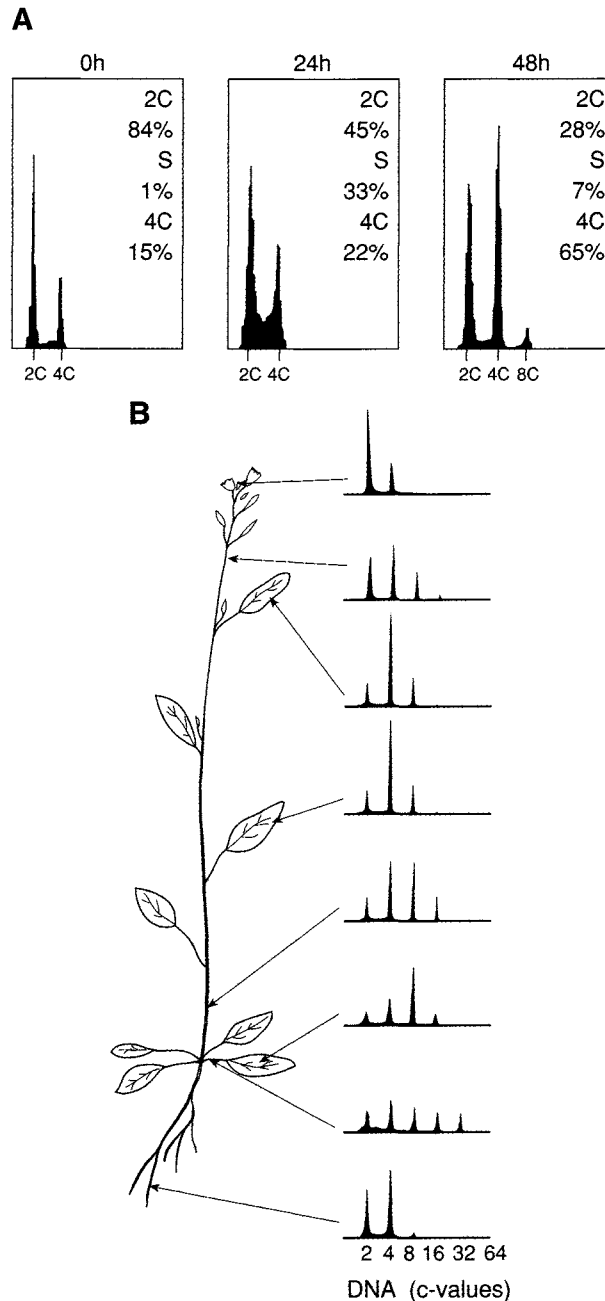


FIGURE 15-5 Nuclear DNA content measured by flow cytometry. (A) Nuclear DNA content in freshly harvested leaf mesophyll protoplasts from *Petunia hybrida* cultured for 48 h. Nuclei at 0 h show mostly 2C DNA content, whereas the majority at 48 h have progressed to G2 and show 4C content. From Trehin *et al.* (1998). (B) Ploidy distribution for nuclei isolated from different parts of a diploid *Arabidopsis thaliana* (cv. Bensheim) plant. Nuclei were isolated by chopping the tissues and were purified by differential centrifugation before measurement of DNA content. Note that mature plant tissues/organs can have up to 32, even 64, C DNA content. With permission from Bergounioux *et al.* (1992), ©1992 Munksgaard International Publishers Ltd, Copenhagen, Denmark.

Whole cells can also be used for measuring the DNA content of nuclei, but in this case, corrections have to be made for the contribution from mitochondrial and chloroplast DNA. Flow cytometry is used for measuring various other things. For example, proportions of protoplasts that have acquired a new cell wall (this is done by staining with calcofluor, which specifically stains β 1,4-linked glucans and emits a blue fluorescence when excited by light at 345 nm), and measuring chlorophyll fluorescence in isolated plastids, etc.

Expression of cyclins and CDKs: As mentioned earlier, many plant CDKs are homologues of Cdc2 or CDC28 in yeast and contain the PSTAIRE motif (see Chapter 2). Antibodies prepared against this motif allow a determination whether the cdc2 protein is being expressed and, if so, by how much. Indeed, the very first studies on cdc2 protein expression in plants utilized this method (John *et al.*, 1989). Northern blots or tissue prints using gene-specific cDNA probes may be used to check the expression of a specific cyclin or CDK in relation to the G1/S or G2/M transition. Constructs that consist of the promoter of a specific CDK or a cyclin gene and the coding sequence of a reporter gene, such as β -glucuronidase (*GUS*, the gene is referred to as *uidA*), may be used to transform whole plants, and the expression of the reporter gene monitored in regions where divisions are expected. Cells and protoplasts obtained from these transformed plants and kept in suspension culture may also be used.

Hormonal effects: Hormones may be used in combination with any or all of the just described to check their effects on specific stages of cell cycle or expression of specific cyclins or CDKs. If cell cultures are used, precautions are taken to deplete the cells of their endogenous hormones, or render them "quiescent," by withholding hormones and nutrients, such as sucrose, for some time (usually 48 h) before resuspension in media containing combinations of sucrose and different hormones.

2.1. Systems Differ in Their Requirements for Hormones for Induction of Cell Division

2.1.1. Lateral Roots

As explained in Chapter 14, lateral and adventitious rooting is promoted by auxins, but inhibited by cytokinins. These conclusions are further supported by the site-specific expression of cyclin or *cdc2* genes. In one of the first studies using *cdc2* or cyclin probes, it was shown that *cdc2* mRNA levels in pea roots were increased significantly within 10 min of exposing the roots to IAA (50 μ M). The *cdc2* protein content and activity continued to rise to 25 h and were significantly higher than in the water control. Roots incubated for 24 h in zeatin riboside showed much reduced levels of *cdc2* protein in the elongation zone correlating with inhibited lateral root formation.

Arabidopsis plants were transformed with a construct consisting of the promoter of a mitotic cyclin gene (*Cyc1At*) fused to the reporter gene, *GUS*. Treatment of the transgenic plants with auxins (IAA, NAA, or 2,4-D) induced the formation of many laterals, which also showed strong *GUS* activity (Fig. 15-6). Similar results were obtained with constructs containing the promoter of *cdc2aAt* (the *Arabidopsis* homologue of *cdc2*) and *GUS*.

2.1.2. Suspension Cell Cultures

Cells in suspension culture, like calli, have an obligate requirement for an auxin and a CK for cell division

(although as explained in Chapter 4, "habituation" may result in isolation of hormone-autonomous lines, which synthesize sufficient endogenous hormone). If constructs containing the promoter of either a CDK or a cyclin gene and a *GUS* coding sequence are used to transform plants, mesophyll protoplasts can be obtained, which can then be used to check the effects of hormones on specific stages of cell division, includ-

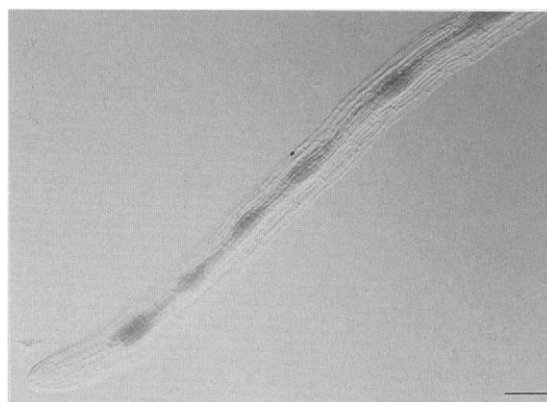


FIGURE 15-6 Expression of a mitotic cyclin gene (*Cyc1At*) during auxin-induced lateral root formation. *Arabidopsis* plants were transformed with a construct consisting of the promoter of *Cyc1At* and the coding sequence of the gene for β -glucuronidase (*Cyc1At::GUS*). Transformed plants were treated with 10^{-6} M NAA. *GUS* activity, localized histochemically, is seen primarily in the pericycle, especially at the sites of lateral root primordia. Roots were cleared with lactophenol and photographed using differential interference contrast optics. Bar: 100 μ m. From Ferreira *et al.* (1994).

ing expression of the respective CDK or cyclin. Data from two related studies utilizing *cdc2aAt* or *Cyc1At* promoters from *Arabidopsis* and *GUS* are shown in Fig. 15-7. Transformed mesophyll protoplasts treated with an auxin (NAA) together with a cytokinin, benzyladenine (BA), strongly enhanced [3 H]thymidine incorporation (S phase, DNA synthesis). As shown by *GUS* activity, they also strongly induced *cdc2* and *Cyc1At* expression. Treatments with auxin or BA alone promoted *cdc2* expression without significantly

increasing DNA synthesis and gave poor expression of the cyclin. These data suggest that while both auxin and cytokinin are necessary for DNA synthesis and for the expression of the mitotic cyclin (*Cyc1At*), the expression of *cdc2* can proceed by auxin or cytokinin alone.

The suspension cell culture system is being used by several groups of researchers to determine the effects of added auxin and/or CK on G1/S transition or G2/M transition and whether any specific cyclin or a CDK is induced by either hormone. The specific details are still unclear, but some conclusions are beginning to emerge. It appears that there is a specific requirement for cytokinin for the entry of cells into G1/S transition. In *Arabidopsis* cells in culture, *CycD3*, a G1 cyclin, is specifically induced by zeatin or BA at concentrations as low as $10^{-3}\mu\text{M}$ (Fig. 15-8A). *In situ* hybridizations show that *CycD3* mRNA is abundant in meristematic tissues, such as shoot apex, young leaves, lateral buds, procambium, and differentiating vascular tissue in maturing leaves, all regions of high meristematic activity. *CycD3* mRNA is expressed before the onset of S phase, as shown by both [3 H]thymidine incorporation into DNA and expression of the histone H4 gene (Fig. 15-8B). The authors proposed that the *CycD3* gene is transcriptionally activated by cytokinin and that such activation is essential for the G1/S transition. It is believed that the CDK activated by cyclin D drives the release of the E2F transcription factor from its complex with a retinoblastoma-type protein. Such release then results in the expression of proteins necessary for DNA replication and expression of mitotic cyclins that can drive DNA synthesis during the S phase (see Section II,3.3 in Chapter 2).

Cytokinin is also essential at the G2/M transition. In *N. tabacum* BY-2 cells, which are autonomous for cytokinin synthesis, the G2/M transition is the time when cells sharply accumulate cytokinin. In *N. plumbaginifolia* cells, which have a stringent requirement for cytokinin for mitosis, cells arrest in the G2 phase unless supplied cytokinin. In arrested cells, *cdc2*-like protein (CDK) is inactive with heavy Tyr-15 phosphorylation; it requires dephosphorylation by a Cdc25-type phosphatase for entry into mitosis (see Section II,3.2 in Chapter 2). Thus, cytokinin seems to be required at the G2/M transition to stimulate the tyrosine dephosphorylation of the CDK/cyclin B complex, an event that results in the release of catalytic activity of the protein kinase, which is able to drive the key mitotic events of chromosome condensation, preprophase band disassembly and nuclear envelope breakdown. Whether cytokinin is also involved in the expression of the gene encoding *CycB* in the complex is not known.

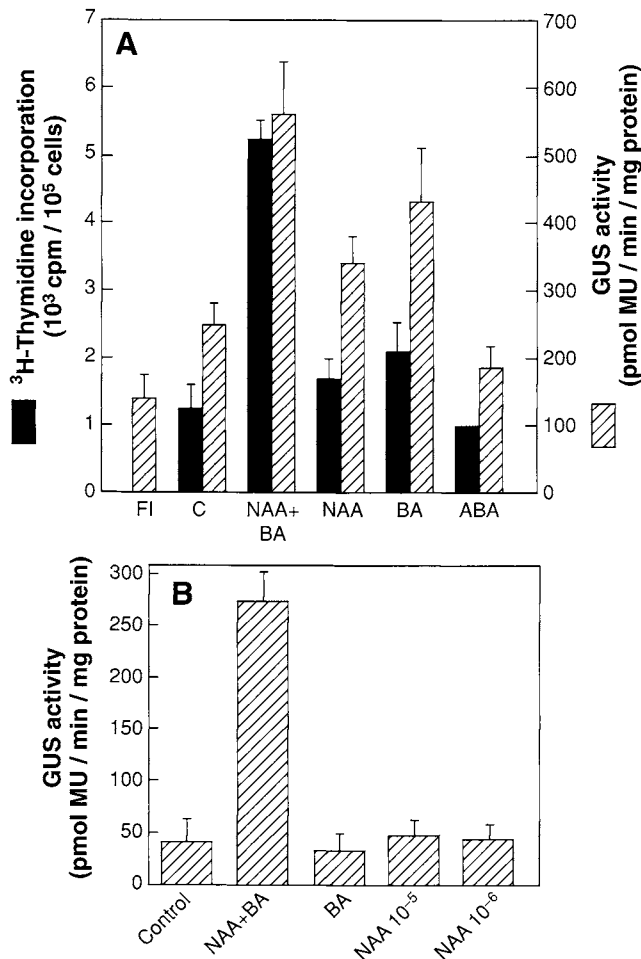


FIGURE 15-7 Expression of a CDK (*cdc2aAt*) and a mitotic cyclin (*Cyc1At*) in leaf protoplasts obtained from transformed tobacco plants. (A) Plants transformed with the *cdc2aAt* :: *GUS* construct. Freshly isolated protoplasts were cultured for 3 days in medium containing [3 H]thymidine and $1.0\mu\text{M}$ of the indicated hormones, except for the NAA + BA treatment (NAA, $5.0\mu\text{M}$ + BA, $1.0\mu\text{M}$. FI, freshly isolated protoplasts; C, control, protoplasts cultured without hormone. Adapted from Hemerly *et al.* (1993). (B) Plants transformed with the *Cyc1At* :: *GUS* construct. Protoplasts were cultured as before but without thymidine treatment. Hormonal concentrations are indicated. Standard errors are shown. GUS activity is expressed in terms of methyl umbelliferone (MU). Adapted from Ferreira *et al.* (1994).

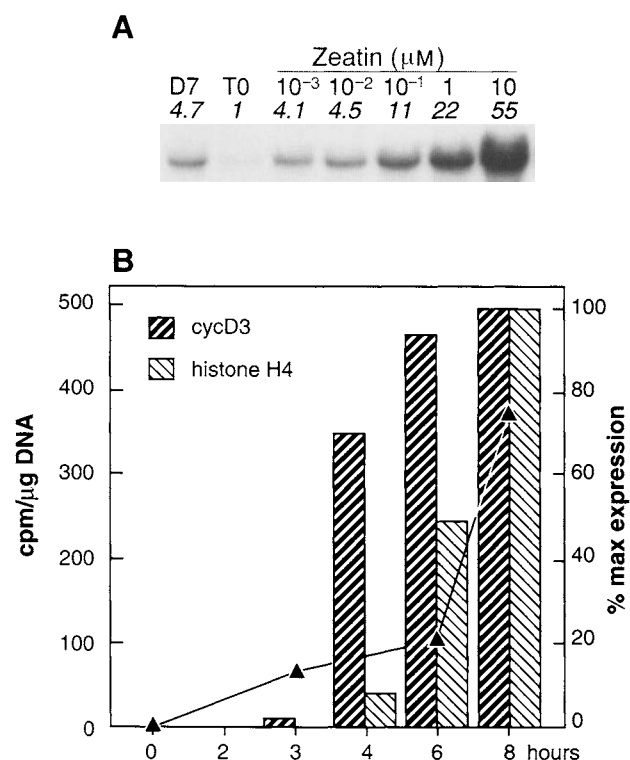


FIGURE 15-8 *Arabidopsis* CycD3 gene expression. (A) CycD3 mRNA induction by zeatin in suspension cell cultures. Early stationary cells [day 7 (D7)] were washed and incubated in a medium with sucrose but without auxin and cytokinin for 24 h. They were sampled before induction [$T = 0$ (T0)] and 4 h after treatment with increasing concentrations of zeatin. Quantitation of CycD3 mRNA relative to $T = 0$ is shown in italics. (B) CycD3 and histone H4 expression and DNA synthesis in *Arabidopsis* cells reactivated from quiescence by sucrose addition at 0 h. These suspension cultures were derived from calli from *Arabidopsis* plants transformed in a special manner to express CycD3 constitutively, i.e., without the addition of exogenous CK. [3 H]Thymidine incorporation was measured at indicated times (triangles, left axis) and plotted together with bars showing the quantitation of CycD3 and histone H4 levels (bars, right axis). The bars are plots of the hybridization intensity as determined by Northern blots. With permission from Riou-Khamlichi *et al.* (1999), © 1999 American Association for the Advancement of Science.

Auxin is also necessary for cell cycle progression, although its specific roles are unclear. An auxin treatment is essential for the entry of quiescent, stationary phase cells into the cell cycle. Auxin also seems to be essential for the G2/M transition, as cells can block at both of these points if auxin is withdrawn. Auxin regulation of the cell cycle may involve more diverse mechanisms, including a general stimulation of growth.

Figure 15-9 summarizes our current information on auxin and cytokinin regulation of cell cycle progression. Both hormones are essential, but their requirement for cells in suspension culture will vary with species, cell type, and culture conditions. For cytokinin, the transition that is most dependent on ex-

ogenous supply will also vary with the levels of endogenous cytokinin, cyclin D concentration, and Cdc2a/cyclin B activity.

2.1.3. Stem Segments

Stem segments of deepwater rice elongate in response to simulated flooding or GA₃ treatment, and such elongation involves both increased cell division and cell elongation (see Fig. 15-2). In stem sections treated with GA₃, transcripts of a CDK, *cdc2Os-2*, increased within 1 h of GA treatment and of two B-type mitotic cyclins, *cycOs1* and *cycOs2*, shortly thereafter (Fig. 15-10). The induction of *cdc2Os-2*, identified as a G1/S phase CDK, was specific to GA because the expression of another CDK, *cdc2Os-1*, was not affected by GA. Rice stems are a nonsynchronized system. While some cell divisions were seen as early as 4 h after GA treatment, the rate of DNA synthesis increased rapidly between 4 and 6 h after GA treatment, followed by a sharp increase in the percentage of cells accumulating in G2. These cells would have undergone mitosis even later. Flow cytometry confirmed that a significant proportion of cells progressed from G1 through S to G2 over 4–8 h after GA treatment. Recently, two other genes encoding a CDK, *cdc2Os-3*, and an A type cyclin, *CycA1;1*, have been shown to be coordinately expressed in response to GA-treatment. These results are significant because this is the first demonstration that GAs might be involved directly in DNA synthesis and in regulating cyclin or CDK components in both G1/S and G2/M transitions.

Stem segments from mature, nonelongating regions of hypocotyls/epicotyls of dicot seedlings can also be induced to divide under the influence of exogenous auxins (2,4-D), but they have not been investigated for the induction of cyclins or CDKs.

2.2. Cytokinins May Regulate the Cell Cycle at Other Steps

In addition to their effect on the induction of cyclins and/or CDKs, cytokinins have been implicated at other steps as well. Replication of DNA in eukaryotic chromosomes occurs at multiple sites, in a series of replication units called replicons. Each replicon has a point of origin where replication starts and proceeds in opposite directions in the two strands. This bidirectional replication has been shown by [3 H]thymidine labeling and DNA fiber autography, as well as by electron microscopy of isolated and shadowed DNA. The size of the replicon is usually constant for a given species. In one study, [3 H]thymidine was used to label DNA; it could be shown that following a single application of benzyladenine ($4.5 \times 10^{-5} M$) to intact shoot

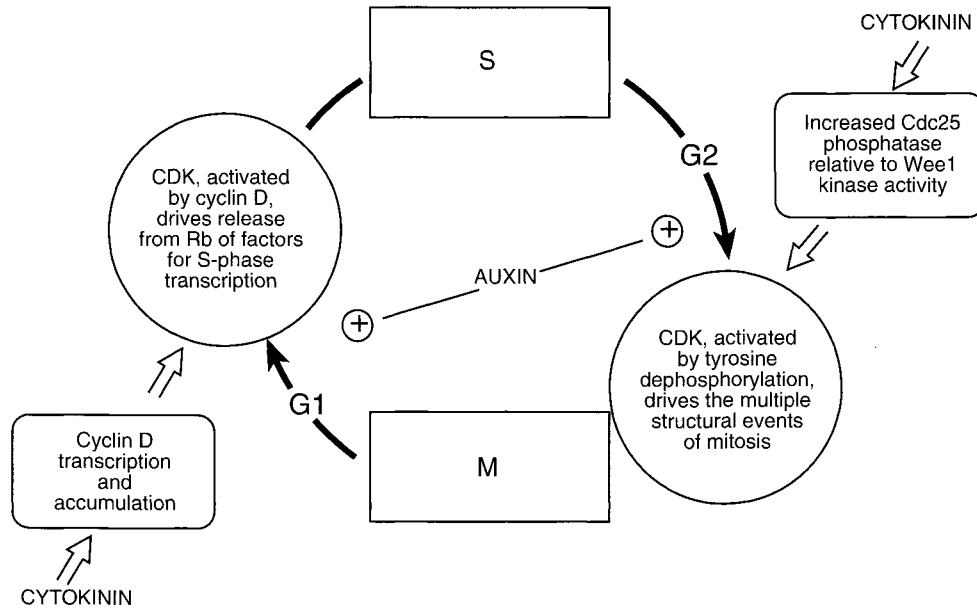


FIGURE 15-9 Hormonal regulation of cell cycle progression. Cytokinin stimulates the transcription and accumulation of cyclin D during the G1 phase. The increased CDK/cyclin activity in turn drives the release of factors essential for S-phase transcription. During the G2/M transition, cytokinin stimulates the activity of the Cdc25 phosphatase-type protein, resulting in tyrosine dephosphorylation of CDK/cyclin, and the release of kinase activity that brings about mitosis-associated events. The specific role of auxin is unclear, but it is required for both transitions. Rb, retinoblastoma-type protein; Wee1 kinase is the enzyme responsible for Tyr-15 phosphorylation in yeast. Courtesy of Pete John, Australian National University, Canberra.

apices of *Lolium* (a monocot) or reproductive axes of tomato (a dicot), the replicon size decreased from about 22 to about 12 μm in both species. It was proposed that CKs activated “latent” replication origins and thus decreased the replicon size, which resulted in a shortened S phase and a more rapid cycling of cells.

Among related processes, CKs have been reported to stimulate rRNA production. RNA polymerase I (pol I) mediates the transcription of rRNA. In one study, the promoter from a pol I gene was fused to a reporter gene, and the construct was used to transform *Arabidopsis* plants. Various plant hormones were applied to the transformed plants, and their effects on rRNA transcription were checked by GUS expression. Only cytokinin (kinetin) stimulated GUS expression, within 1 h of treatment, whereas an application of GA_3 , ABA, auxin, or ethylene had no detectable effect.

2.3. Inhibition of Cell Division by Absciscic Acid

ABA has been known to inhibit cell division in fronds and roots of *Lemna minor*. In *Arabidopsis* plants transformed with a *cdc2aAt* promoter: GUS construct, treatment with ABA (10 nM to 1.0 μM) results in lack of expression of GUS and lack of [^3H]thymidine incorporation in nuclei of lateral root tips. A similar

decrease in the expression of GUS activity and inhibition of cell division after ABA treatment is seen in isolated protoplasts from transformed tobacco mesophyll cells (see Fig. 15-7A). As mentioned in Chapter 2, there are specific regulatory proteins that inhibit the kinase activity of cyclin/CDK complexes. The first plant gene for such an inhibitor protein, ICK1 (for interactors of Cdc2 kinases), has been cloned in *Arabidopsis*. The gene is specifically induced by ABA, and along with its induction, there is a decrease in Cdc2-like histone H1 kinase activity. Furthermore, the ICK1 protein binds specifically *in vitro* to Cdc2a and CycD3 proteins. These results may explain the inhibitory effect of ABA on cell division.

3. HORMONES MAY BE INVOLVED IN DETERMINATION OF PLANE OF CELL DIVISION

Do hormones regulate planes of cell division? As shown in Chapter 2, plant cells determine their planes of division by the localization of the preprophase band of microtubules (PPBs) considerably before mitosis. Factors regulating the arrangement of PPBs are a mystery and little is known about the role of hormones in

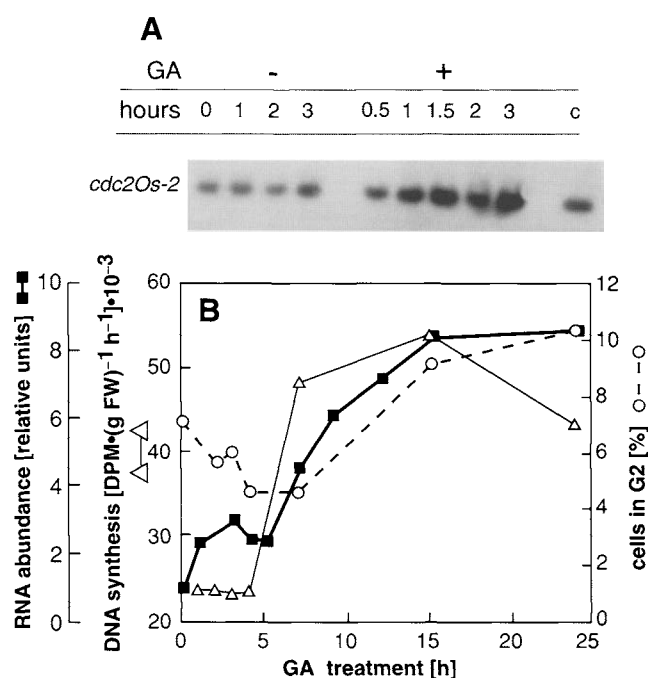


FIGURE 15-10 Cyclin and CDK expression in the intercalary meristem of GA₃-treated rice internodes. (A) Transcript levels of a CDK, *cdc2Os-2*. The increase in transcript levels occurs within 1 h of GA treatment. Reverse transcription polymerase chain reaction was used to allow the detection of transcripts present in low abundance. The lane labeled "c" represents the amplified product from the *cdc2Os-2* cDNA clone. (B) Kinetics of expression of *cycOs2* that encodes a B type cyclin, rate of DNA synthesis, and accumulation of cells in G2 following GA treatment. *cycOs2* transcript levels were plotted from radioactivity associated with RNA bands probed with a ³²P-labeled antisense riboprobe. Note that the rise in the rate of DNA synthesis is followed shortly by a rise in the percentage of cells accumulating in G2. With permission from Sauter *et al.* (1995), © 1995 Blackwell Science Ltd.

that process. Nonetheless, gibberellins have been implicated in promoting transverse divisions in hypocotyl tissue. In a short hypocotyl mutant (*hyp2*) of *Nicotiana plumabiginifolia*, it was shown that treatment with GA₃ reduced the frequency of longitudinal divisions and promoted transverse divisions, thus increasing the length and reducing the diameter of roots.

4. PEPTIDE GROWTH FACTORS AND HORMONES

In mammalian systems, cell divisions are promoted by various chemicals, including peptide growth factors. These substances, known as mitogens (agents that promote mitosis), signal quiescent cells to enter the cell cycle. A special phosphorylation/dephosphorylation cascade, known as the mitogen-activated

protein kinase (MAPK) cascade, mediates the transmission of an external signal to the nucleus, leading to the synthesis of cyclins specific to the G1/S transition and phosphorylation of substrate proteins by their cognate CDKs. In plants, the various elements in the MAPK cascade have been identified (see Chapter 25). A growth-promoting peptide factor has also been identified. Attempts are being made to link the effects of auxins or cytokinins to the induction of MAPK cascades or to the induction of genes encoding peptide growth factors.

5. SUMMARY OF HORMONAL INDUCTION OF CELL DIVISION

Since the mid-1980s, there has been considerable progress in our understanding of the cell cycle in plants and its hormonal regulation. Three hormones, auxins, cytokinins, and gibberellins, individually or in combination, regulate events in the cell cycle by stimulating or inhibiting the expression of *cdc2* homologues or cyclins. The role of brassinosteroids in cell division is still unclear. Many of the cyclins identified to date in plants are B-type, mitotic cyclins. Genes for several D-type cyclins involved in the G1/S transition have also been identified, and one of them, *CycD3* in *Arabidopsis*, has been shown to be specifically induced by CKs. Auxins and cytokinins are both essential for DNA synthesis and mitosis in callus tissue or cells in suspension culture; however, the specific roles of the two hormones in the passage of cells over G1/S or G2/M transitions are still unclear. It is also unclear whether GAs interact with other hormones in stimulating *cdc2* or cyclin expression. Other questions that need resolution include the interaction among auxins and CKs and the expression of *cdc2* homologues and/or cyclins during the initiation of lateral root primordia or lateral bud outgrowth. It is also unclear how ABA inhibits cell division, whether it counters the positive effects of mitosis-promoting hormones, or whether it acts in the mitotic process itself. A direct effect is indicated by its induction of an *ICK* gene in *Arabidopsis*.

Plant cells lack a defined START or restriction point in their cell cycle, and quiescent parenchyma cells can enter the S or M phase from G1 or G2, respectively, without, at least as yet, any clearly defined signal. Nonetheless, multiple signals, both developmental and environmental in origin, must impinge on control points in late G1, or in G2, to commit plant cells to enter the S phase or the M phase, respectively. Wounding and environmental stimuli, such as exposure to light and cold temperature, are known to induce cell division. However, it is not known whether these environ-

mental stimuli act independently, via elements similar to MAPK cascades, to trigger cyclin or CDK synthesis or whether their effects are mediated *via* hormones.

Before leaving this section, an important point must be emphasized. GAs do not seem to bring about cell division in roots, and no effect of GA₃ on *cdc2aAt* expression in *Arabidopsis* roots was observed. However, GAs stimulate cell division in aerial stems and petioles and induce both *cdc2* and cyclin expression in internodes of deepwater rice. It is likely that the cell division-specific activities of other hormones are also tissue and organ specific.

SECTION II. CELL GROWTH

1. HORMONAL REGULATION OF CELL GROWTH

Chapter 2 showed that plants exhibit two main types of growth; tip growth as seen in pollen tubes and root hairs and overall diffuse growth, which is best seen in axial organs, such as stems, roots, petioles, flower pedicels, and grass coleoptiles. As far as we know, the tip growth of pollen tubes (and presumably root hairs) is not directly affected by phytohormones. Pollen grains are a rich source of brassinosteroids (see Chapter 9), and there are a few reports of BRs affecting pollen germination and tube growth, but the details are not clear. Tip growth, therefore, is excluded from further consideration in this chapter. In contrast, overall diffuse growth is very much affected by three classes of hormones: auxins, gibberellins, and BRs. That such growth in intact plants is mediated by endogenous hormones is shown by the use of hormone synthesis mutants and synthesis inhibitors. Application of exogenous hormones to whole plants and cut segments provides further confirming evidence.

1.1. Evidence That Hormones Are Involved in Growth in Intact Plants

GA synthesis mutants, such as *le* pea, are unable to convert GA₂₀ to the physiologically active GA₁ in their shoot tissues and remain dwarf. If exogenous GA₁ is supplied, they are restored to the normal wild type (see Chapter 7). If normal plants are grown in the presence of a GA synthesis inhibitor, such as AMO-1618, they are much reduced in height, an inhibition that is corrected by an exogenous supply of physiologically active GAs.

Brassinosteroids bring about cell/organ elongation in aboveground parts. Mutants deficient in BR biosyn-

thesis are genetic dwarfs, which are restored to wild type by the exogenous application of brassinolide or castasterone, but not IAA or GA (see Chapter 9).

Similar data are not available for IAA because as yet there are no known IAA synthesis mutants or synthesis inhibitors.

While the use of genetic mutants and synthesis inhibitors provides compelling evidence that hormones are involved in natural growth, supporting evidence is provided by many other types of experiments.

1.2. Gibberellin (GA)-Induced Growth in Stems

Germinated lettuce seedlings kept in light show a short hypocotyl, but if supplied with exogenous GA₃ (or GA_{4/7}) show considerable elongation of the hypocotyl, 400–500% over water control, in 72 h (Fig. 15-11A; see also Fig. 5-3). The response is shown almost exclusively by the hypocotyl, not by the roots. It is also a strictly GA-mediated response; use of the IAA antagonist PCIB has no effect on growth, which indicates that IAA is not involved. If the seedlings are grown in dark, there is very little significant difference in hypocotyl length between the water control and the GA₃-treated material. Furthermore, if dark-grown seedlings are transferred to light, there is a sudden and dramatic arrest in elongation growth (Fig. 15-11B). These data suggest that there is enough endogenous GA in the dark to sustain elongation growth, but that its synthesis or supply is inhibited in light. Hence the requirement for exogenous GA in light.

GA₁ has been shown to be the predominant biologically active GA in lettuce hypocotyls. It is also the GA responsible for the elongation growth of (dark-grown) hypocotyls because the inhibition of 3β-hydroxylase activity by prohexadione-Ca (see Chapter 7) is alleviated by GA₁, but not by GA₂₀. Moreover, the levels of GA₁ drop by up to 70% on exposure of lettuce seedlings to white light.

Similar GA-induced elongation, with some variations, is known from many other systems, both intact seedlings (e.g., pea epicotyls, Azuki bean hypocotyls) and cut stem segments. Cut segments offer some advantages in that (1) growth can be studied *in vitro* and (2) the system can be manipulated experimentally. Thus, cut segments may be used with DNA synthesis inhibitors to demonstrate whether there is a component of cell division or to test the effects of pH changes or different cations on cell/organ growth. Systems that have been well investigated include lettuce hypocotyl sections, young internodes in stem segments of oat (*Avena*), and deepwater rice (*Oryza sativa*). Reference to deepwater rice was made earlier (see Section I,1.1.2,

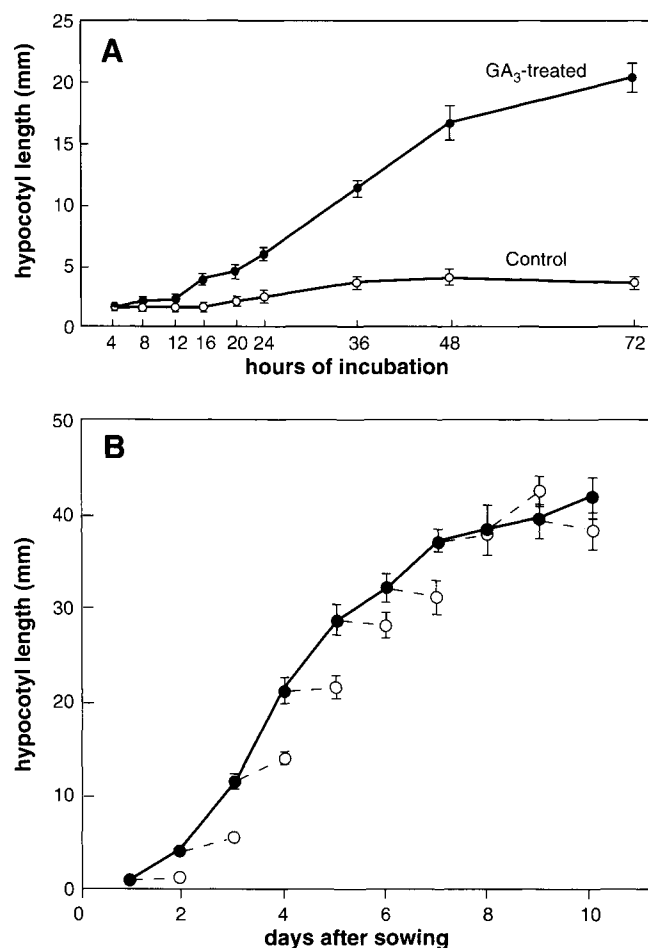


FIGURE 15-11 GA-induced growth of lettuce (*Lactuca sativa* cv Grand Rapids) hypocotyls. (A) Kinetics of growth in light with exogenous GA. Moistened seeds were given a brief exposure to light and were then germinated in the dark. After 24 h they were selected for uniformity, given GA₃ (1.0 μ M), or kept in water (control) and grown for 72 h in light. Each point represents the mean hypocotyl length for 10 seedlings \pm standard error. From Sawhney and Srivastava (1974). (B) Inhibition of hypocotyl elongation by white light. Lettuce seedlings were grown in the dark and, at indicated times, were transferred to white light. Each point represents the mean length \pm standard error of 20–30 assay plants. \bullet —, in the dark; --- \circ under white light. From Toyomasu *et al.* (1992).

Fig. 15.2). Growth of oat stem segments by GA and the effect of GA concentration on growth are shown in Fig. 15-12.

1.3. Auxin-Induced Growth in Cut Segments of Grass Coleoptiles or Dicot Stems

Coleoptiles are cylindrical organs that ensheath the first leaf and shoot apex in grass seedlings. They help in the emergence of the first leaf (and shoot apex) by protecting it during its passage through the soil. On

emergence, the first leaf breaks through the coleoptile tip. Coleoptiles grow by cell division and cell enlargement until they are about 5 mm long, but their subsequent growth is almost entirely due to cell elongation. Coleoptiles of oat and maize are favorite materials for study; they are harvested when they are about 5 cm long, the apical 4–5 mm are discarded, and 5- or 10-mm segments are obtained from the subapical region. The cut segments are usually kept in water or buffer solution for a few hours to deplete the segments of endogenous IAA before external application of the auxin. IAA, as well as the synthetic auxins, 2,4-D and NAA, have been used. Subapical segments of hypocotyls/epicotyls of dicot seedlings (e.g., bean, soybean, mung bean, Azuki bean, zucchini, pea) are also used following essentially the same protocol. The auxin-induced growth of cut stem or coleoptile segments follows a biphasic curve. The initial rise in the growth rate after a lag period is duplicated by weak acids or buffers at an acidic pH, but the second rise is typical of auxin action and can be sustained for many hours if an energy source such as sugar is provided (Fig. 15-13). The lag period varies between 10 and 15 min, depending on tissue; similarly, the rate of growth achieved and the time interval between the first and second peaks vary.

Although most work with auxin-stimulated growth has centered on cut stem or coleoptile segments, there is some evidence that IAA may promote elongation growth in intact plants. In one study, IAA was supplied in a controlled manner, on demand, *via* a wick to the first two internodes of light-grown pea seedlings. It could be shown that there was an enhancement of the growth rate when the IAA supply was turned on and a decline when it was turned off. Moreover, the enhancement was greater in the GA-deficient dwarf pea than in the normal tall pea.

1.4. Brassinosteroid (BR)-Induced Growth

As mentioned in Chapter 9, brassinosteroids are essential for the elongation growth of stems. Mutants deficient in BRs are dwarfs in light or in dark and are not rescued by GAs or auxins. Moreover, an exogenous application of BRs, such as brassinolide or castasterone, promotes the growth of young intact stems in light; in dark-growth material the effect may not be as significant. Thus, BRs, like GAs, are involved in countering the inhibitory effect of light on the elongation growth of young stems. BRs, like IAA and GAs, also promote the elongation growth of cut stem sections. The kinetics of growth in soybean (*Glycine max*) epicotyls after BR treatment shows a lag period of about 45 min, much longer than that for IAA (see Fig. 9-2, Chapter 9).

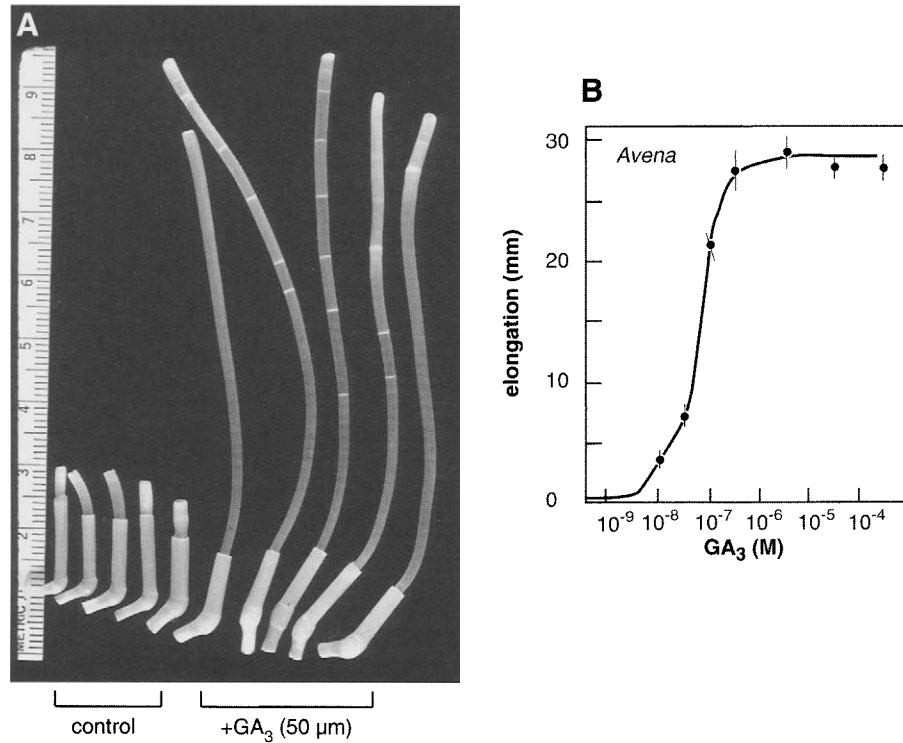


FIGURE 15-12 GA-induced growth of oat (*Avena*) stem sections. (A) Stem sections grown for 48 h in darkness with sucrose (100 μM) and without (control) or with 30 μM GA₃. Courtesy of Peter Kaufman. (B) Relationship between concentration of GA₃ and growth of the stem sections. From Adams *et al.* (1973).

1.5. Indoleacetic Acid, GA, and BR Interaction

The examples given earlier were chosen to show that single hormones are involved in growth, but there are examples where elongation growth is promoted by both IAA and GA (e.g., cucumber and soybean hypo-

cotyls, pea, and Azuki bean epicotyls). Some studies suggest that GAs might be more involved in promoting the growth of young-growing tissues, whereas IAA may be able to promote the growth of somewhat older tissues. For example, in cut segments from the apical 20 mm of cucumber hypocotyls, both IAA and GA stimulated the growth of the youngest 5-mm segment,

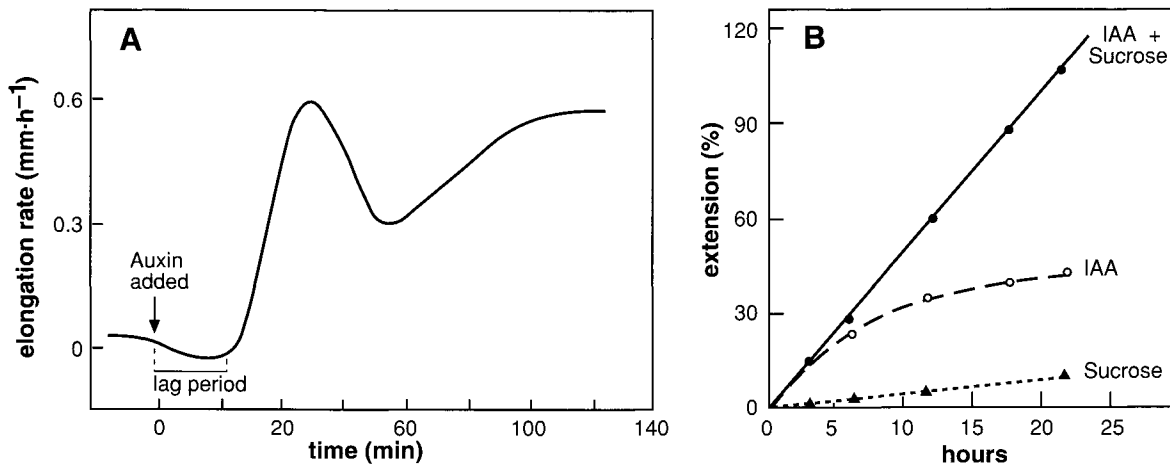


FIGURE 15-13 Kinetics of auxin-induced growth for cut segments. (A) Biphasic response to auxin (2,4-D) in soybean hypocotyl segments. Growth rate rises after a lag period of 10–15 min. The first rise can be duplicated by weak acids, but the second rise is characteristic of auxin-induced growth. From Vanderhoef *et al.* (1976). (B) IAA-induced growth of oat coleoptiles with and without added sugar. From Cleland (1995) with kind permission from Kluwer.

but only IAA stimulated the growth of the basal 5-mm segment. Furthermore, as judged by the difference in the amount of growth between water control and IAA-treated material, the basal segment was more sensitive to IAA-induced growth than the apical segment.

Additional data indicate that the signaling pathways for auxins, GAs, and BRs cross-connect at some point as far as the elongation response is concerned. The *sax1* mutant of *Arabidopsis* is deficient in endogenous BRs (for *sax1*, see BR interaction with other hormones, Chapter 24). In this mutant, inhibition of root elongation by auxin or ABA occurs at lower concentrations than in the wild type (i.e., the mutant is hypersensitive to auxins or ABA in the inhibition of root growth). The mutant also does not respond to exogenous GA₃ for hypocotyl growth in light, unlike other BR synthesis mutants. However, application of 24-epi-BL restores the mutant to a normal response to auxin and ABA in root growth and to GA in hypocotyl growth. These data indicate that the SAX1 protein might be an important common intermediary in auxin-, GA-, and BR-induced elongation responses of stems and roots.

There has been some speculation in the past that GAs promote the growth of stems and petioles *via* IAA, and not by themselves. Evidence from GA synthesis (or response) mutants and GA synthesis inhibitors clearly indicates that GAs promote elongation growth in their own right, not *via* IAA. Similarly, brassinosteroids promote growth in their own right.

1.6. Hormone-Induced Growth Is Time and Tissue Specific

The following sections consider the hormone-mediated extension growth of axial organs in greater detail, but before leaving this section it is important to emphasize two points.

i. Hormone-induced growth is time specific. It is seen only if the organs are in a developmentally competent state for growth. After cessation of elongation or if the growth phase is past, as in old internodes in the stem, provision of the hormone does not bring about a resumption of growth. Also, if the organ has not yet entered the growth phase, an exogenous hormone may have no effect.

ii. Hormone-induced growth is tissue or organ specific. For instance, GAs, BRs, and IAA promote elongation growth in axial aboveground organs. Auxins inhibit root elongation at all but very low concentrations (usually 10^{-8} M or less). BRs also inhibit root growth. At very low concentrations, auxins, GAs, and BRs may promote root growth.

2. CHARACTERISTICS OF ELONGATION GROWTH IN AXIAL ORGANS

2.1. Tissue Tension and Role of Epidermis

Axial organs, such as stems, petioles, and coleoptiles, are composed of thousands of cells and several different tissue types with differing cytological and wall characteristics. All of these cells must grow (or be stretched) in unison if the organ is to elongate. It has been known for a long time that, in a growing organ, there is strong tissue tension (Julius Sachs described it as "Gewebespannung" more than a hundred years ago) produced by the expanding force of the inner tissues, which is balanced by the contracting force of the epidermis (plus, in some cases, one or two subepidermal layers). "Inner tissues" refer to the cortex in stems and petioles and to the mesophyll in coleoptiles; for purposes of this discussion, vascular tissues are omitted from consideration. The tension is best seen in axial organs that are growing rapidly. If a longitudinal split is made in an axial organ, such as stem, the split halves immediately bend outward because the epidermis contracts, whereas the internal tissues expand. If the epidermis is peeled from the organ (this is done easily in some materials, such as maize coleoptile), the epidermal strips contract in length, even in air, while the internal tissues elongate if placed in water (Fig. 15-14). These spontaneous changes indicate that the length of the intact turgid segment depends on a balance between two opposing forces: the tensile stress of the epidermis and an internal pressure, which is equivalent to the turgor of the inner tissues.

The epidermis, thus, has a role not only in mechanical support, but also in regulating the rate of elongation of an axial organ. The epidermis is suited for both these functions by the structure of its outer tangential wall (OTW). These walls are often quite thick, up to 10 times as thick as the inner tangential wall (ITW) or the radial walls. Moreover, as mentioned in Chapter 2, they show a cross-polylamellate structure, i.e., the cellulose fibrils in successive lamellae alternate between a predominantly longitudinal or transverse orientation. Such cross-polylamellation of OTW is seen in electron micrographs of epidermal cells and has been reported by numerous authors (Fig. 15-15).

It follows that in these preparations, the innermost, or the most recently deposited, wall fibrils will occasionally be seen in transverse or, at other times, in oblique or longitudinal orientations. In any one cell, at one time, only one orientation predominates, but different cells in the whole organ may show different

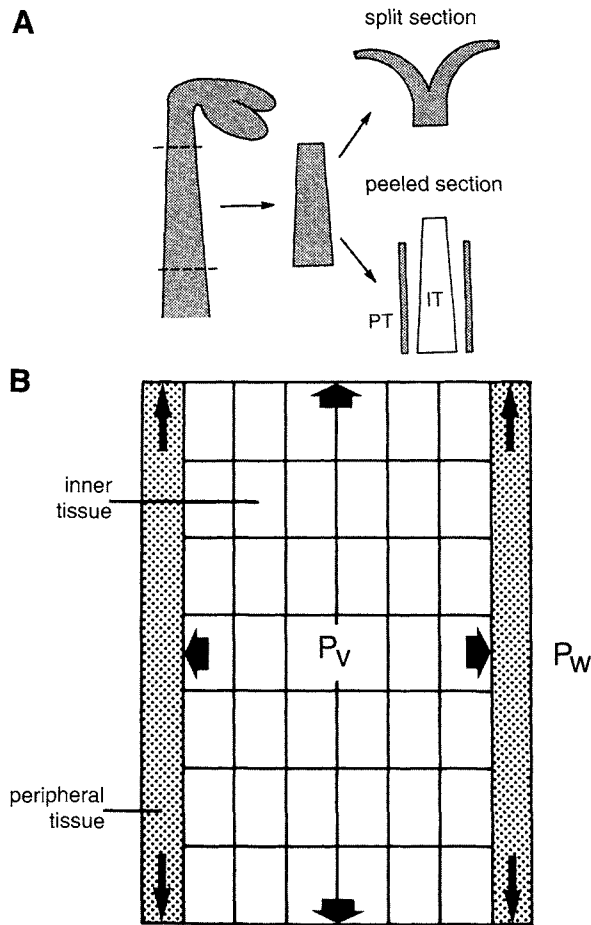


FIGURE 15-14 Two classical experiments and a scheme illustrating that the expansive force of the inner tissues in an axial organ is balanced against the tensile stress of the epidermis. (A) If a rapidly growing stem segment is split longitudinally, the two split halves bend outward because the inner cells expand, while the epidermis contracts (top). If the epidermis (peripheral tissue, PT) is peeled from a stem segment, the epidermal strips immediately contract, even in air, whereas the inner tissues (IT) elongate if placed in water (bottom). (B) In an axial organ, the expansive force or the turgor pressure (P_v) of the inner tissues is held in check by the peripheral tissue (mostly the outer wall of epidermis), which is under longitudinal tension (P_w , wall stress). Modified from Kutschera (1987).

orientations. This is indeed what is seen. Figure 15-16 shows replicas of the inner surface of the OTW of epidermal cells, and it is clear that in different cells, the orientations of innermost wall fibrils may be transverse, oblique, or longitudinal with respect to the long axis of the cell.

A diagrammatic representation of the cross-polylamellate wall is shown in Fig. 15-17. Three different stages are shown with different orientations of the innermost wall fibrils next to the plasmalemma. The cross-polylamellate structure of the OTW is ideally suited for both the mechanical support and the restraining function of the epidermis.

2.2. Orientations of Cortical Microtubules and Cellulose Fibrils in the Innermost Lamella

As shown in Chapter 2, orientation of the innermost, newly deposited cellulose fibrils parallels that of cortical MTs. Also, that newly deposited, cellulose fibrils determine the directionality of cell growth. While the latter conclusion is easily confirmed for single cells or filamentous organisms, such as *Chara* or *Nitella*, it is unclear in a multicellular organ, such as a stem or coleoptile, whether the directionality of growth is determined by the innermost cellulose fibrils in cortical or epidermal cells. To complicate matters, the orientations of MTs and innermost cellulose fibrils change depending on the rate of organ growth, i.e., whether the organ is growing rapidly, is growing slowly, or has stopped growing. For valid conclusions, therefore, it is important to examine tissues that are confirmed to be clearly growing or not growing and to stipulate which cells and walls are being examined.

Several materials and techniques have been used. Some authors have marked stems with India ink to determine which internodes are rapidly growing or, alternatively, have stopped growing (control); some have used hormonal treatment to boost the elongation of hypocotyls/epicotyls or young internodes of deep-water rice while the controls are left in water; some have used red light, which allows growth of pea epicotyls, and blue light, which stops growth almost instantaneously; and some have used *le* and *d5* mutants of pea and maize, respectively, and boosted them to elongate by GA. Still other studies have utilized sub-apical segments of pea epicotyls made to expand radially by ethylene. Because one sees only a small fraction of the tissue in electron micrographs, immunofluorescent light microscopy or confocal laser scanning microscopy (CLSM) has been used increasingly to visualize orientations of MTs in whole cells.

Despite the diversity of plant materials and techniques used, the picture that emerges is remarkably consistent. Cortical MTs and innermost wall fibrils in the inner tissues (cortical or mesophyll cells) are always transverse (or helical with a shallow pitch) in organs that are elongating rapidly (Figs. 15-18 and 15-19). If the growth rate is slow or if growth has stopped, cortical MTs and innermost wall fibrils in these cells may still remain transverse or may show an oblique or even a longitudinal orientation.

In contrast to the walls of cortical (or mesophyll) cells, the OTW of epidermal cells show considerable variation, which is augmented by the fact that these walls normally deposit cellulose lamellae in alternating longitudinal and transverse orientations. In organs

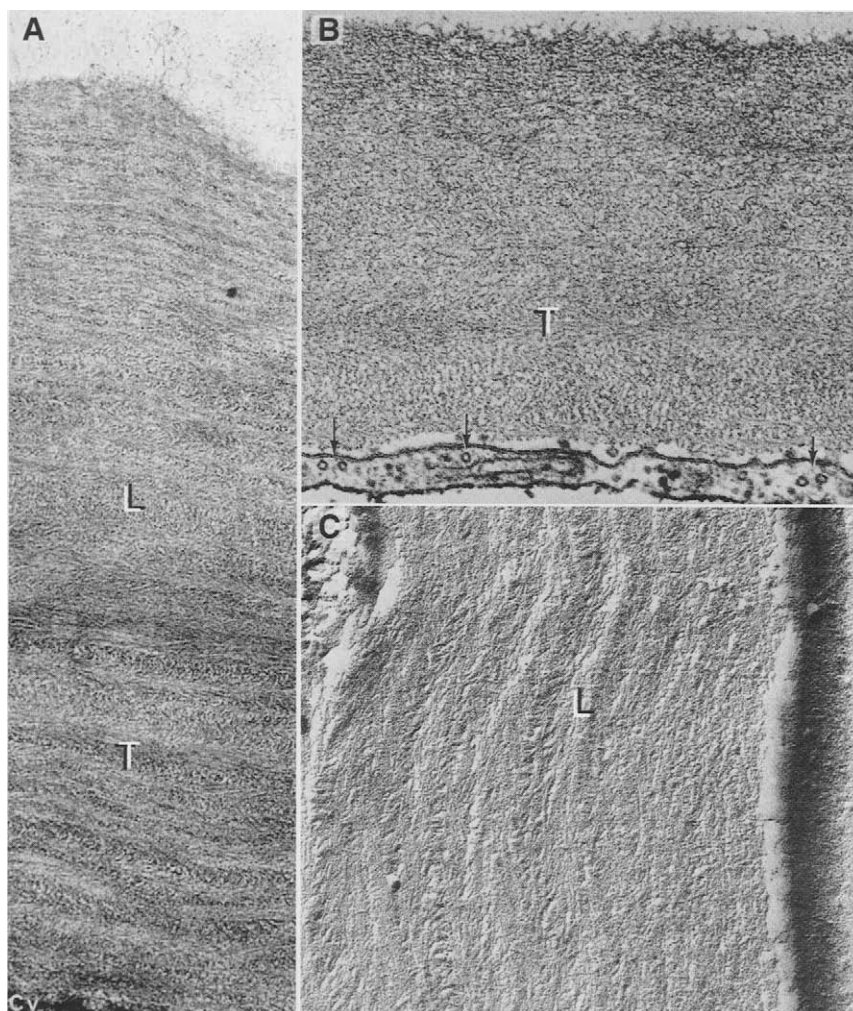


FIGURE 15-15 Cross-polylamellate structure of the outer tangential walls (OTW) of epidermal cells. (A) Transverse section of the OTW of an epidermal cell in *Rumex*. (B and C) Transverse and longitudinal sections of the OTW of epidermal cells of lettuce (*Lactuca sativa*) hypocotyl treated with GA_3 for 72 h. In all cases, the walls show a polylamellate structure with transversely running lamellae alternating with longitudinally running lamellae. In transverse sections, longitudinally running fibrils (L) appear cut in cross-sectional view, whereas transversely running fibrils (T) appear in their long orientation. In the outermost part of the wall lamellation may be lost. In A, cy=cytoplasm. In B, both microtubules (arrows) and innermost cellulose fibrils have a longitudinal arrangement. Materials for A and B were fixed in glutaraldehyde/ OsO_4 , embedded in plastic, thin sectioned, and stained with lead and uranium salts. Material for C was fixed and sectioned as above, then the plastic was removed by treatment with sodium methoxide, and the preparation was shadowed with platinum-palladium. A from Chafe and Wardrop (1972); $\times 24,000$. B and C from Sawhney and Srivastava (1975); B, $\times 48,000$; C $\times 50,000$.

that are growing normally, MTs (and, by inference, innermost wall fibrils) in epidermal cells show all three arrangements, transverse, oblique, and longitudinal, although the proportion of cells with transverse or longitudinal MTs changes with the status of cell/organ growth. Figure 15-20 shows histograms of proportions of epidermal cells in two locations in cucumber hypocotyl: one just below the cotyledonary node and the other 3–4 mm below which was slowing down. In the control, the apical region shows most

cells with an oblique orientation of MTs, whereas in the basal region, a proportionally greater number of cells show a longitudinal orientation. In GA_3 -treated material, in contrast, much the higher number of cells show a transverse arrangement of MTs, and those with longitudinal orientation are far fewer. If organ growth is arrested by ABA treatment, the proportion of cells with longitudinal MTs rises. Similarly, in another study on maize coleoptiles that had stopped elongating, MTs against the OTW of most epidermal cells

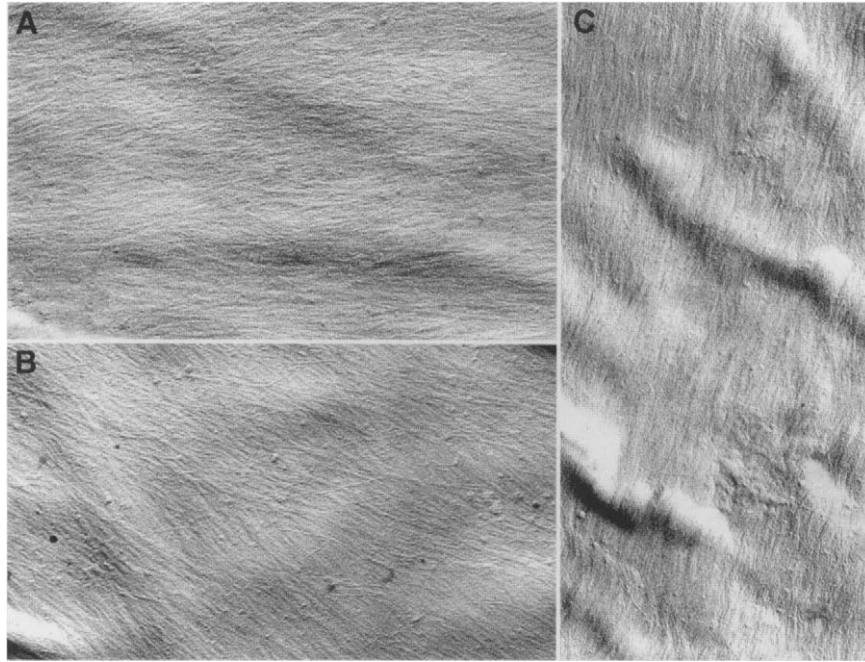


FIGURE 15-16 Replicas of the inner surface of the OTW of epidermal cells from the epicotyl of Azuki bean seedlings. In some cells, the orientation is transverse (A), oblique (B), or longitudinal (C). The organ axis is parallel to the length of the page. To prepare these photographs, the inner surface of the OTW was exposed by fracturing, and a carbon replica was made and shadow cast by metal. From Takeda and Shibaoka (1981).

showed a longitudinal orientation, which was quickly changed to transverse when the coleoptile segments were boosted to elongate by auxin treatment.

Radial or anticlinal walls (AW) and the inner tangential wall (ITW) of the epidermal cells show a mixed situation. In rapidly elongating organs, these walls usually show a transverse arrangement of MTs and innermost wall fibrils; in cells that have stopped elongating, they may be oblique to longitudinal.

The picture that emerges from these observations, and from the demonstration of tissue tension between epidermis and inner tissues (see Fig. 15-14), is that the inner, cortical, or mesophyll cells in an axial organ provide the driving force for elongation growth. These cells consistently show a transverse orientation of their cortical MTs and innermost wall fibrils, especially so when the organs are boosted to elongate by exogenous hormone application. Epidermal cells with

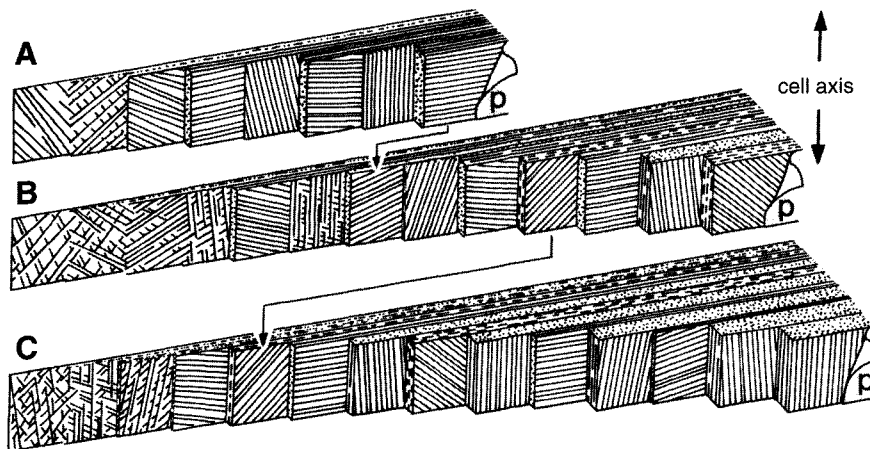


FIGURE 15-17 A diagrammatic representation of the polylamellate structure of the OTW of epidermal cells. Note the transverse, oblique, or longitudinal orientation of the innermost wall fibrils next to the plasmalemma (P). From Takeda and Shibaoka (1981).

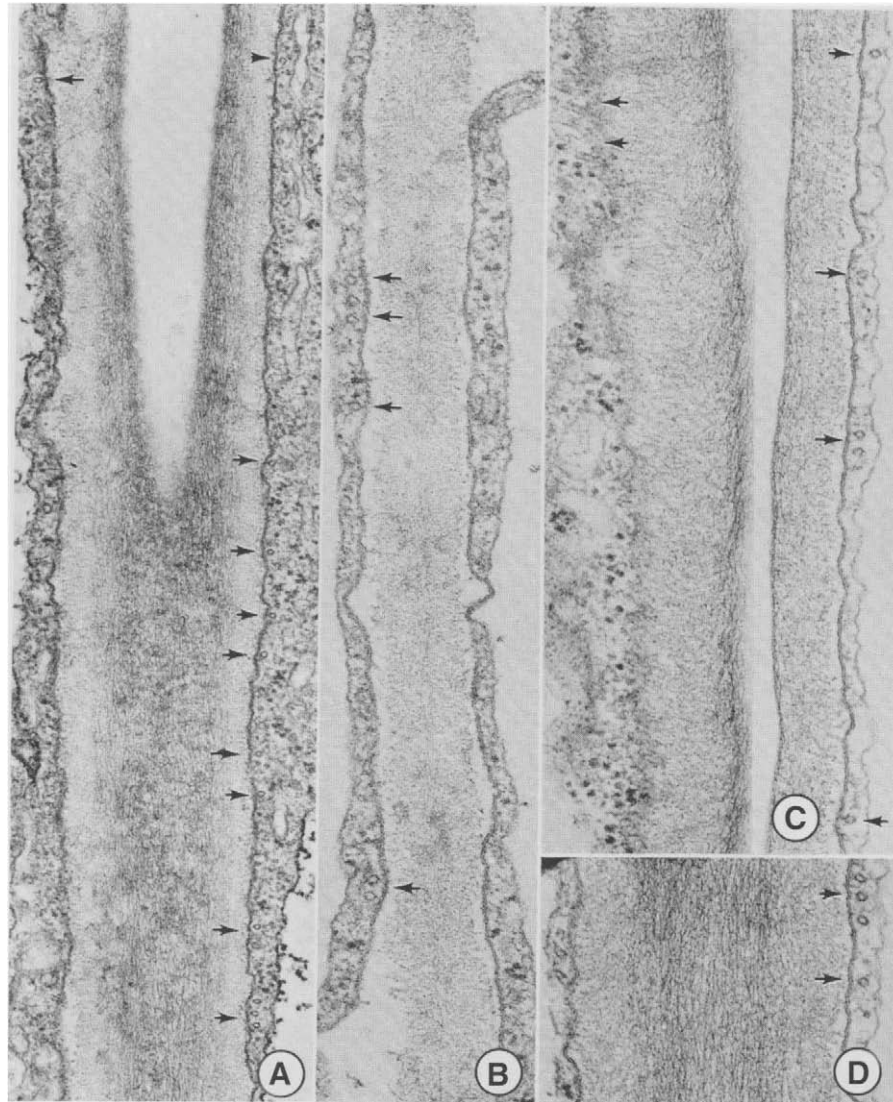


FIGURE 15-18 Orientations of innermost wall fibrils and MTs in common tangential walls of cortical cells in lettuce hypocotyls boosted to elongate rapidly by GA_3 . All are longitudinal sections. (A) 24 h GA_3 ; (B) 48 h GA_3 , and (C and D) 72 h GA_3 . In C, the cells have separated along the middle lamella, and the oldest parts of the wall about the intercellular space, which has been trimmed for mounting. An intercellular space also appears in the upper part of A. In all figures, Microtubules (arrows) and innermost wall fibrils have a transverse arrangement with respect to the long axis of the cell. A, $\times 35,600$; B–D, $\times 53,300$. From Sawhney and Srivastava (1975).

their cross-lamellate structure in the outer tangential wall serve to regulate the rate of such growth. In organs growing very rapidly, most epidermal cells show a transverse arrangement of MTs and innermost wall fibrils in the OTW, whereas in slower growing organs or in those that have ceased elongation, a proportionally greater number of cells may show a longitudinal orientation of MTs and innermost wall fibrils.

2.3. The Orientation of Microtubules Can Change very Quickly

Clive Lloyd and associates at John Innes Centre in Norwich, UK, have followed changes in MT orientations in living epidermal cells by immunofluorescence and CLSM. Their studies show that MTs against the OTW can change within minutes from being transverse to oblique/longitudinal as growth stops and

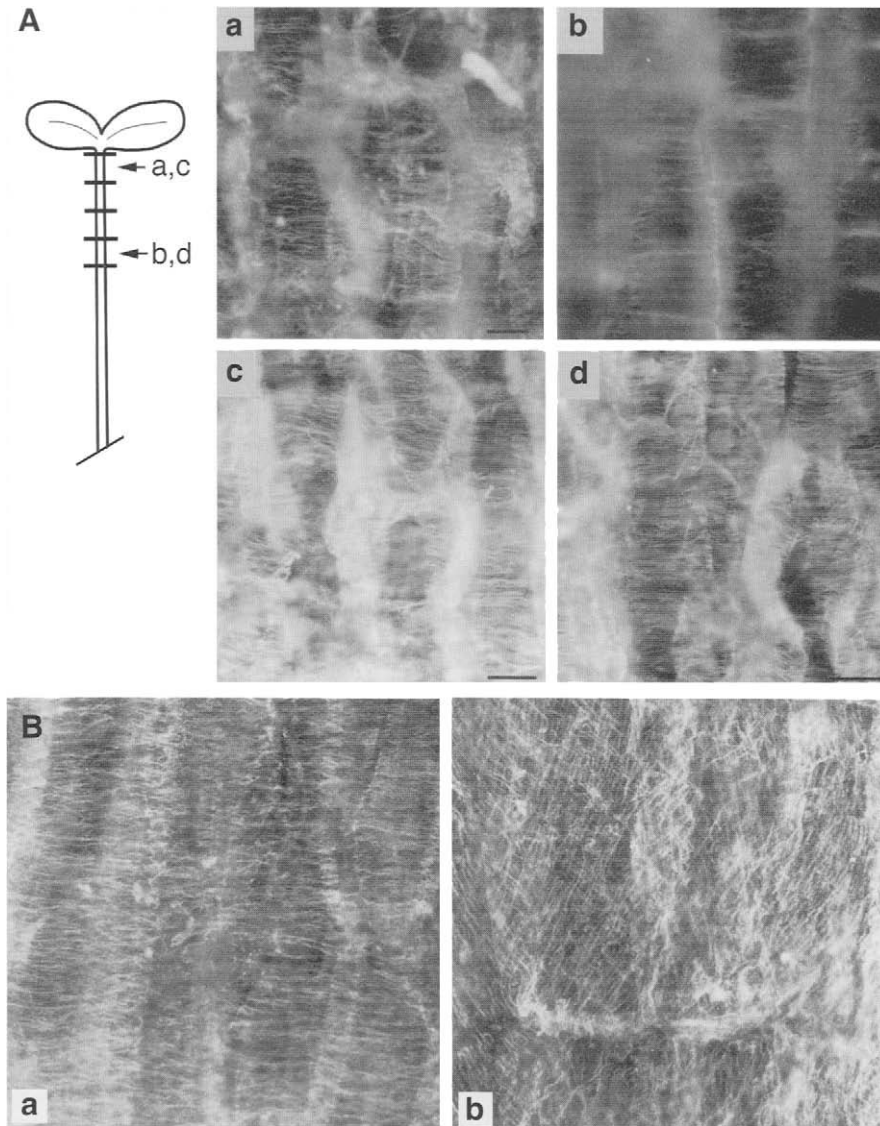


FIGURE 15-19 Orientations of microtubules in cortical cells as shown by immunofluorescent microscopy. (A) Control (a and b) and GA₄ (10 µg/plant)-treated (c and d) cucumber hypocotyls. Samples were taken from two regions: 0–1 (a and c) and 3–4 (b and d) mm below the cotyledonary node; see diagram of the seedling on the left. Cells from both regions show transversely arranged MTs. With permission from Ishida and Katsumi (1992), ©1992 The University of Chicago Press. (B) Cortical MTs in subepidermal cells from an internode of *Pisum sativum*. (a) Cells from seedlings that were induced to grow by red light irradiance and (b) cells from the same region after growth had been stopped by irradiance with blue light. From Laskowski (1990).

from being longitudinal to transverse if sections are placed in a GA₃ solution (Fig. 15-21). It has often been observed that MTs show one orientation against the OTW, but other orientations against the radial wall or the ITW of the same epidermal cell. Because CLSM allows optical sectioning of the material, a series of images taken at different levels in the cell and integrated can then be rotated by computer imaging. Data from these observations show that the same MTs may show one orientation against the OTW and may con-

tinue with a different orientation against one of the radial walls.

3. RADIAL GROWTH OF AN AXIAL ORGAN

Treatment of an axially elongating organ with ethylene causes an arrest of longitudinal growth, and its swelling (growth in diameter). Such arrest in longitudinal

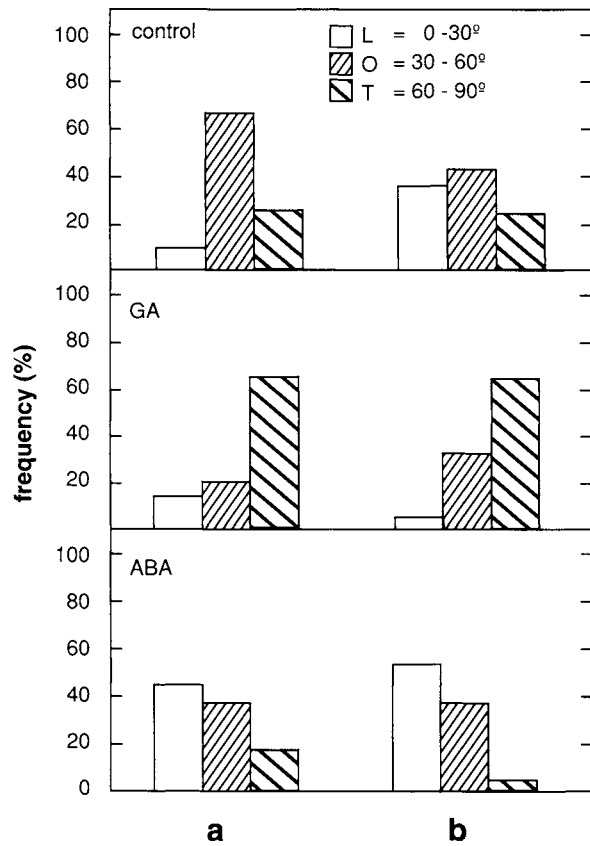


FIGURE 15-20 Histograms showing proportions of cells with transverse, oblique, or longitudinally arranged MTs against the OTW of epidermal cells in cucumber hypocotyls. Cells were scored, after immunofluorescent staining of MTs, at two locations; 0–1 mm below the cotyledonary node (a) and 3–4 mm further down (b) (see Fig. 15-19A). Data are presented for seedlings kept in water (control) or given GA₃ (10 µg/plant) or ABA (10 µg/plant). MT orientations refer to angle ranges of 0 to 30 (L, longitudinal), 30–60 (O, oblique), and 60–90 (T, transverse) with respect to the long axis of the cells. With permission from Ishida and Katsumi (1992), ©1992 The University of Chicago Press.

growth and swelling is also seen if these organs are supplied with an excess of IAA (which results in the production of ethylene) and in mutant plants that overproduce ethylene (see Chapters 11 and 21). Individual cells, both in the cortex and in the epidermis, expand radially and, in extreme cases, become isodiametric. MTs and innermost wall fibrils in these cells progressively acquire a longitudinal or even random arrangement (Fig. 15-22). Similarly, if rapidly growing cells are treated with chemicals that depolymerize MTs, such as colchicine or amiprophos-methyl, the innermost cellulose fibrils in cortical cells are laid in a random fashion and the cells become isodiametric. It is important to emphasize that these results of ethylene or colchicine treatment are seen only if the cells are growing rapidly. In cells that

have stopped growing, depolymerization of MTs (by colchicine) or their randomization (by ethylene) has little evident effect on orientation of innermost cellulose fibrils.

There are other instances also where the axial growth of an organ is inhibited and radial growth is promoted. Examples include the development of tubers in potato, storage roots in dahlia and radish, and storage leaves in onion bulbs. Whereas the hormonal and environmental stimuli for these developmental phenomena vary, the structural basis for constituent cells to become spherical is the same. In each case, MTs become disposed randomly or longitudinally, and the innermost cellulose fibrils reflect a similar arrangement.

4. HOW DO HORMONES CAUSE CELL EXPANSION?

As explained in Chapter 2, several conditions must be satisfied before elongation or radial growth of axial organs can occur. These conditions include a loosened wall (for all the constituent cells of the organ) and an uptake of water and, if sustained growth has to be obtained, provision for new synthesis of wall materials and the maintenance of turgor pressure. At the same time there must be regulatory control over the orientations of MTs and thus indirectly over the orientation of newly deposited cellulose fibrils.

Growth under normal conditions proceeds in such a way that the expansive force created by turgor and the loosened wall is kept in balance by wall synthesis. This growth involves endogenous hormones whose synthesis and supply are strictly regulated. Growth induced by the supply of exogenous hormones shifts the balance, at least temporarily, in favor of the expansive force; hence, the spurt of growth observed so frequently in hormone-treated materials. However, it should be remembered that the exaggeration of growth by exogenous hormones, while allowing a dissection of the process of growth, also introduces artifacts that may not be present under normal circumstances.

How do auxins, GAs, and BRs bring about elongation growth? Do they affect water uptake, wall loosening, or both? Are they involved in the synthesis of new wall materials and membranes? Do they control the orientation of MTs in cortical or epidermal cells and thereby influence the directionality of growth? These are important questions, but we still have only partial answers to them. For historical reasons, even though GA-induced elongation growth

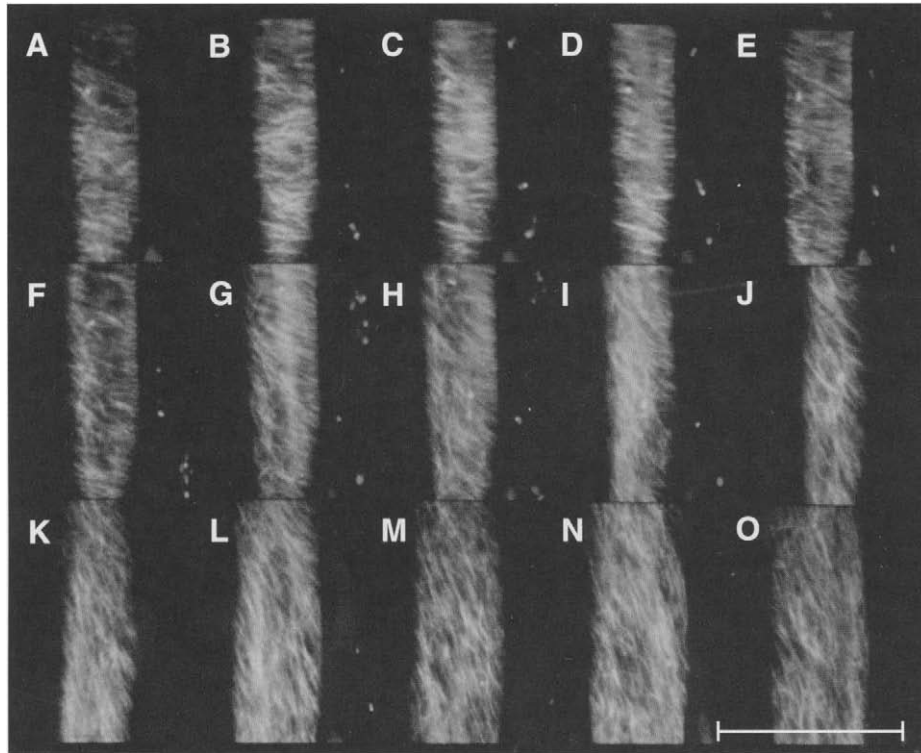


FIGURE 15-21 Change in orientation of cortical MTs under the OTW of a pea epidermal cell from nearly transverse to nearly vertical. Living epidermal cells in longitudinal slices of the second internode of pea seedlings were microinjected with rhodamine-conjugated pig brain tubulin, which fluoresces with light from a laser beam in the CLSM. Successive projections are separated by ca 6-min intervals. Note that the change from nearly transverse (A) to oblique (J) orientation is essentially completed in about 45 min. In the lower row (K–O), there is less dramatic reorganization, as the array of MTs adopts a steeply oblique configuration. Bar: 50 μm . From Yuan *et al.* (1994).

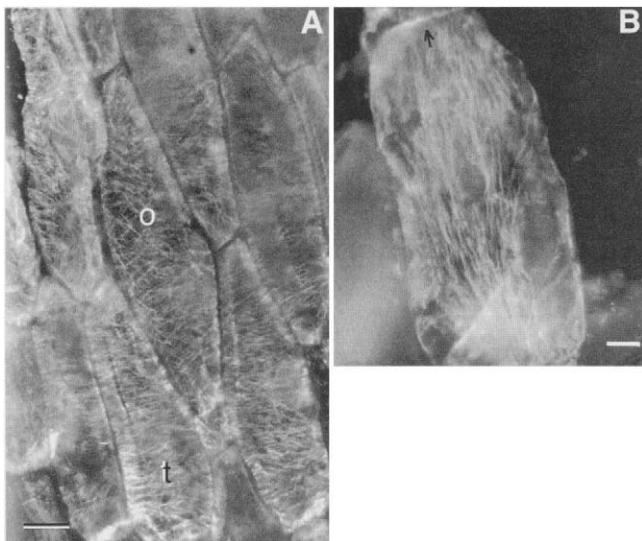


FIGURE 15-22 Micrographs of epidermal cells from mung bean (*Vigna radiata*) hypocotyls treated with ethylene for 1 (A) or 3 (B) h. (A) Some oblique (o) and transverse (t) MTs are still seen, but they are mostly longitudinal in B. Bar: 20 μm (A), 10 μm (B). From Roberts *et al.* (1985).

is usually much larger in magnitude than auxin-induced growth, much of the information on wall loosening and water uptake comes from the auxin-induced growth of cut coleoptile or stem segments. Information from GA-induced growth is relatively meager, and information from BR-induced growth is just beginning to accumulate. In the following, therefore, IAA-induced growth is reviewed separately from GA- and BR-induced growth, although it must be understood that plants, most likely, utilize the same mechanisms and motive force for cell/organ growth, irrespective of whether the growth is brought about by IAA, GAs, or BRs.

4.1. Auxin-Induced Growth

As far as we know, auxin does not increase the osmotic potential of the cell and therefore does not affect water uptake directly. Water uptake in cells is caused by a rise in solute concentration inside the cell (or a rise in osmotic pressure, which is equivalent to a decline in the free water potential, Ψ).

Another possibility is the increased conductance of water from the xylem to cortical/epidermal cells in whole tissues. Studies focusing on these aspects have shown that auxin neither increases the solute uptake by cells nor increases the water conductance. Nonetheless, auxin-induced growth is accompanied by an increased uptake of water, which is correlated with an enhanced uptake of solutes. As a result, the overall difference in osmotic pressure of the cell sap between control vs auxin-induced tissue is minimal.

Auxin is known, however, to cause wall loosening, which in turn may reduce the turgor pressure and thus increase the water potential difference between the outside and the cell interior. A dramatic illustration of wall loosening by IAA is shown in Fig. 15-23 and forms the basis of the so-called "split" bioassay for auxin. If subapical segments of coleoptiles (or epicotyls/hypocotyls) are split lengthwise with the epidermis in place (unpeeled), the split halves bend outward in water, but if they are placed in an IAA

solution, they bend inward. They do so because of the loosening of the OTW of the epidermal cells and the greater apparent growth of the epidermal tissue than that of internal tissues.

How does auxin bring about wall loosening? Before discussing this, it is important to understand how wall loosening or extensibility is measured.

4.1.1. Plastic vs Elastic Extension and Measurement of Extensibility

When a tissue is stretched, it expands, and when the force is released it may come back to its original length or it may come back only partly. If it comes back to its original length, the extension (or deformation) is considered "elastic"; if it does not, that means there has been an irreversible change in length. Growth, which is an irreversible change in volume, involves plastic extensibility; it has nothing to do with elastic extensibility. Elastic (reversible) extension, however, is important in such cellular processes, as stomatal opening and closing, which involve reversible changes in the volume of guard cells. However, to measure growth in isolated stem or coleoptile segments *in vitro*, it is important to be able to distinguish between elastic vs plastic extensibility.

Stretchability of yarns and fibers is of extreme importance to the textile industry; hence, sensitive instruments have been available, which plant physiologists have adapted for measuring the extension of plant segments. One such instrument, an extensometer, is shown in Fig. 15-24A. The plant segment is clamped at one end and a force (load) applied at the other end. When the force is released, the tissue springs back and settles at a certain length. Extensibility is given by the equation: strain (% change in length) divided by stress (force per unit area). The change in length is measured using a linear displacement transducer; stress is a function of the load applied divided by the cross-sectional area of the specimen. The two quantities, elastic vs plastic extension, can be calculated readily from length measurements. Data from an actual experiment are shown in Fig. 15-24B.

Not only fresh stem or coleoptile segments, but also segments that have been frozen, stored, thawed out, and abraded to let solutions permeate are used and give identical results. Plastic extensibility in these segments is sometimes referred to as "creep."

4.1.2. Plastic Extensibility Varies with Age and Environmental Factors

Plastic extensibility varies developmentally. It has been shown in stems and roots that extension growth

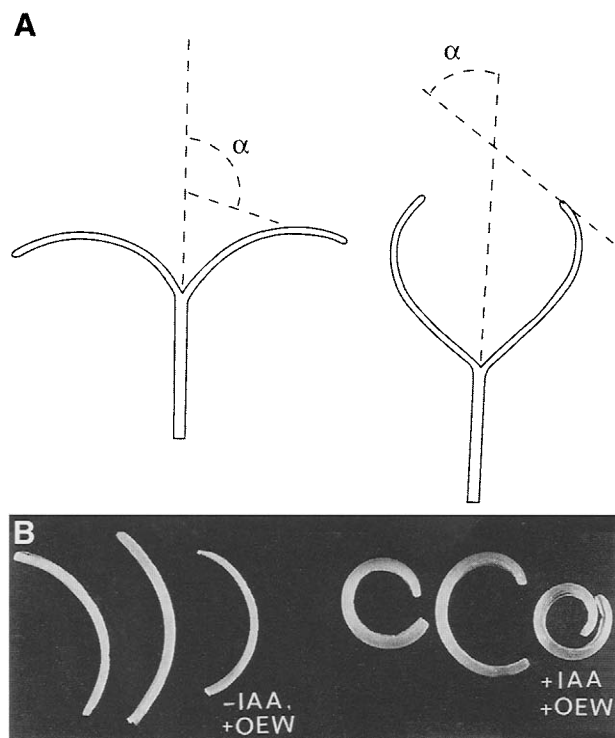


FIGURE 15-23 (A) Schematic illustration of a split bioassay. Split halves bend outward in water (left), but bend inward in a solution of IAA (right). The degree of curvature, which can be measured, provides the bioassay for auxin (the degree of curvature would also vary with the structural characteristics of the OTW). (B) Demonstration of wall loosening by IAA. Subapical segments of maize coleoptiles were split lengthwise and placed in buffer \pm IAA. The outer surfaces of all segments point to the left. From Kutschera *et al.* (1987).

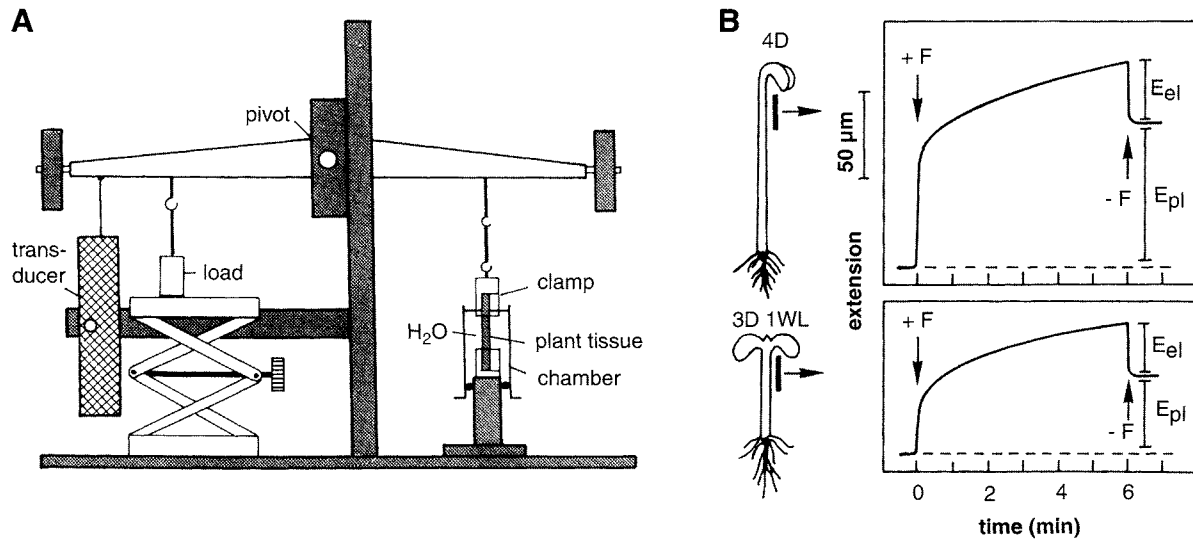


FIGURE 15-24 Measurement of the extensibility of living stem or coleoptile segments. (A) An extensometer has two clamps to hold the segment in place inside a chamber. One end of the clamped tissue is connected to a system that can carry a load and, after a preset time interval, release the load. Extensibility is measured by a linear displacement transducer. The chamber can be filled with water (control) or auxin, or any other test solution. (B) Data from an experiment showing elastic vs plastic extensibility of the subapical region of sunflower seedlings that were grown for either 4 days in darkness (4 D) or 3 days in dark followed by 1 day in white light, $1000 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (3D 1WL). Sections 18 mm in length were excised from the hypocotyls and were cut longitudinally into two halves; one half was fixed between the clamps, immersed in water, and subjected to a constant force of 0.98 N (corresponding to a load of 10 g) for 6 min (+F). After removal of the force (-F), elastic extension (E_{el}) and plastic extension (E_{pl}) were determined as indicated. After Kutschera (1991).

occurs in subapical internodes or regions, but sooner or later, it comes to a stop. This capacity for growth in younger regions is correlated with their greater plastic extensibility than that of older, nongrowing regions/internodes, and this can be shown using an extensometer (see Fig. 15-25).

Plastic extensibility also varies with environmental factors, such as exposure to light, pH changes, presence of cations, nonionizing osmolytes, and hormones. As shown in Fig. 15-24B, plastic extensibility for stem segments from the same subapical region of sunflower hypocotyls is much higher for seedlings grown for 4 days in darkness than for those grown for 3 days in dark followed by 1 day in light. Thus, exposure to light for 1 day changes the extensibility of tissues. Among the cations, K^+ is known to enhance auxin-induced growth, whereas the presence of Ca^{2+} inhibits growth, probably because it forms cross-links between PGA units in the pectins. If a tissue is placed in a solution of nonionizing osmolyte, such as sugars or sugar alcohols (e.g., sucrose, mannitol, polyethylene glycol), auxin-mediated cell elongation is inhibited. In this case, because the water potential outside (Ψ_{out}) may be lower than that inside the tissue (Ψ_{in}), the tissue may actually lose water.

Plastic extensibility is enhanced at low pH, and, very importantly, it is eliminated if the segments are placed in boiling water even for 1 min. Data correlating age of tissues, extensibility under acidic pH, and elimination of such extensibility if tissues are boiled are shown in Fig. 15-25. For Fig. 15-25, subapical regions of cucumber hypocotyls grown in darkness for 5 days were marked into sequential 1-cm segments with India ink, and their growth rate was measured after another 8 h in darkness. It is clear that the apical segment grew the most, while the bottommost segment grew hardly at all. The plastic extensibility of 1-cm segments taken from the same positions, but bathed in buffer at pH 4.5 (Fig. 15-25B) or at pH 6.8 (Fig. 15-25C) showed clearly that growth occurred in acidic pH and correlated with growth of segments *in vivo*, but that in segments kept at pH 6.8, there was little growth and no such correlation. Another very important result is shown in Fig. 15-25D. If the segments are placed in boiling water for as little as 15 s, there is a total loss of plastic extensibility, which suggests that some proteins are involved in plastic (irreversible) wall extension.

Why do older nongrowing parts become nonextensible? We do not know for sure, but the difference in the extensibility of segments of different ages probably

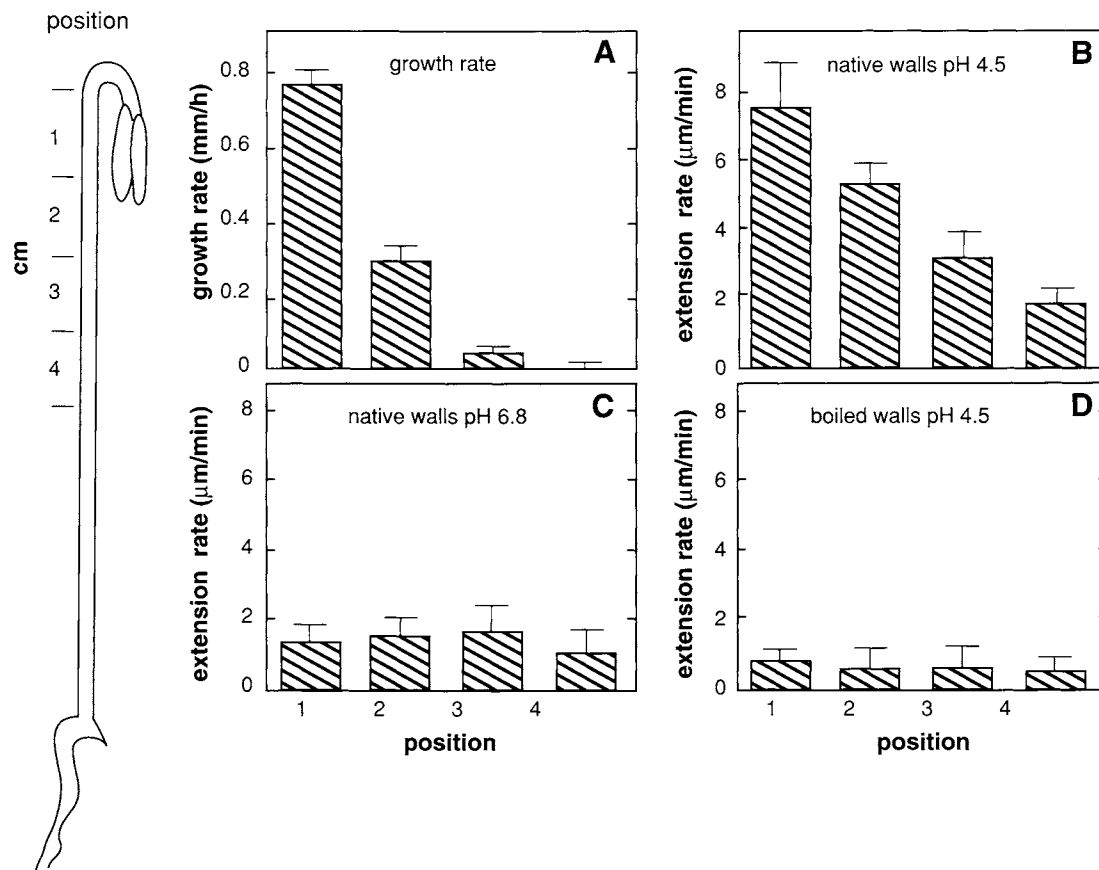


FIGURE 15-25 Distribution of growth *in vivo* and plastic extension of isolated segments of *Cucumis sativus* hypocotyls at different pH and after boiling. Cucumber seedlings were grown in darkness for 5 days, and the subapical region of hypocotyl was marked with ink to denote 1-cm segments (left). (A) Calculated growth rate of segments in intact seedlings after 8 h in darkness. (B to D) The same 1-cm segments were excised at the time of marking, and their plastic extensibility was measured using an extensometer. A constant tensile force of 20 g was applied to the clamped sections, and extension of the walls was measured by a linear variable displacement transducer. During extension, the segments were bathed either in acidic buffer (50 mM sodium acetate, pH 4.5) (B) or neutral buffer (50 mM sodium phosphate, pH 6.8) (C) or, prior to measurement at pH 4.5, were immersed in boiling water for 15 s (D) From McQueen-Mason (1995).

stems from the physical attributes of their cell walls: as the tissues age, the walls become stiffer and nonextensible. As explained in Chapter 2, several factors may play a role in this change: greater hydrogen bonding between hemicelluloses and cellulose fibrils, increased polymerization or enhanced covalent linkage of hydroxyproline-rich glycoproteins, such as extensin, or greater cross-linking of polygalacturonan chains by Ca^{2+} . In cells that deposit secondary walls and lignin, plastic extensibility is completely lost, although elastic extensibility remains.

4.1.3. Auxins Enhance the Plastic Extensibility of Cell Walls in Responsive Regions

That IAA enhances the plastic extensibility of isolated plant segments was first clearly shown by Anton

Heyn at Utrecht, Holland, in the 1930s. His work has been repeated by many others, including Robert Cleland and Daniel Cosgrove in the United States, Ulrich Kutschera in Germany, and Simon McQueen-Mason in the United Kingdom, with better and more delicate instruments, and there is no doubt that auxin treatment of responsive tissues causes an increase in their plastic extensibility. However, how auxin enhances the extensibility of cell walls is not completely understood. Most of our information is derived from *in vitro* experiments; the situation *in planta* is not known. In the following, the major concepts are reviewed briefly.

4.1.3.1. Acidification of the Cell Wall

That wall extension is dependent on pH was first observed by Robert Cleland and associates at

the University of Washington, Seattle, and led to the development of the “acid growth theory” (Rayle *et al.*, 1970). According to the theory, application of auxin causes acidification of the cell wall, which in turn causes wall loosening by activating wall-loosening enzymes. This loosening, associated with net water uptake, brings about an initial growth, which lasts a short time. More sustained growth is obtained if an external energy source, such as sucrose, is provided in addition to auxin—continued growth requires synthesis of new proteins, membranes, and wall materials.

Auxin-induced acidification has been traced to a plasma membrane-based H^+ -ATPase (proton pump), which extrudes protons into the cell wall or apoplast, and whose activity is stimulated by auxin. This activation of H^+ -ATPase occurs within 15–30 min of the auxin treatment and thus coincides with the lag period in auxin-induced growth. It has also been suggested that auxin application causes new synthesis of H^+ -ATPase.

The auxin-induced acidification of the apoplast, by itself, is not sufficient to elicit the growth rates observed. Because boiled wall segments lose the ability for plastic extension, it is thought that growth must also involve the activities of proteins and/or wall hydrolases, which disrupt the cellulose fibril-hemicellulose network (or “load-bearing” polymers, a term used in extensibility measurements) and which have pH optima in the acid range.

4.1.3.2. Wall Hydrolases

In the commonly accepted model of wall architecture, hemicellulose chains are tightly hydrogen

bonded to the surfaces of cellulose fibrils and interconnect them (see Chapter 2). Hydrolases that disrupt these hemicellulose chains would be expected to loosen the cellulose fibrils such that expansion could occur (Fig. 15-26A). Hemicelluloses include xyloglucans (XGs in dicots and many monocots, type I walls) and xylans and mixed-linkage glucans (in grasses, type II walls). Several exo- and endoglucan hydrolases, including endo-1, 4- β -glucanases (EGases, or “cellulases”), xyloglucan endotransglycosylases (XETs), and 1,3-1,4-mixed-link glucanases, have been isolated from the cell walls of a wide variety of plants.

EGases are a large family of enzymes (see Box 15-2). Some EGases have been reported from growing tissues, such as epicotyls or hypocotyls, or cells in suspension culture. The natural substrates for plant EGases are not known, but are thought to be xyloglucans. Although EGases are found in growing tissues and have been implicated in auxin-induced growth for a long time, direct evidence for such a role is lacking. Moreover, the kinetics of EGase mRNA accumulation after auxin treatment argues against a direct role. In some studies, the EGase mRNA required many hours to accumulate, whereas auxin-induced growth had a lag period of ~ 15 min. It is possible, however, that EGases serve other functions in growing organs. For instance, they may cleave XG chains and create more acceptor sites for transglycosylation by XETs (see below)

XETs have the dual function of hydrolyzing a 1,4- β link in a glucan chain and transglycosylating one-half of the cleaved chain to the nonreducing end of another 1,4- β -linked chain. The 1,3-1,4-mixed link glucanases,

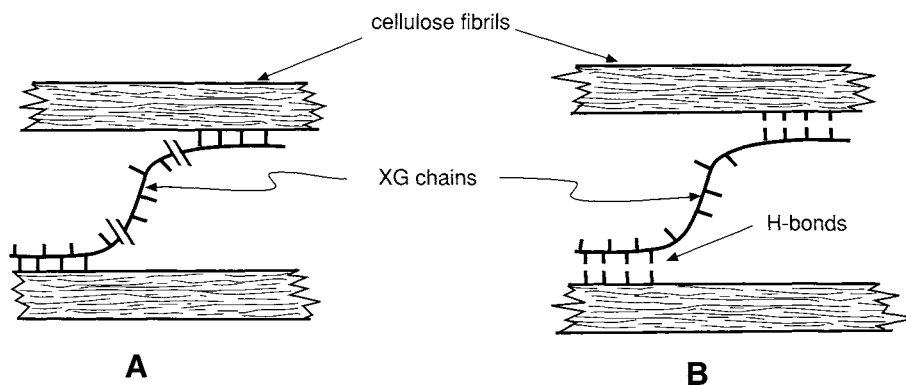


FIGURE 15-26 Models that show how walls can be loosened. (A) A break in the xyloglucan (XG) backbone allows two cellulose fibrils to move apart. Such breaks occur at specific locations in the β 1,4-linked chain and may be caused by XETs. EGases may also cause breaks in XG chains. (B) An XG chain is linked to cellulose fibrils by hydrogen bonds. Expansins, which are soluble proteins resident in the wall, are believed to disrupt these hydrogen bonds, thus allowing two cellulose fibrils to move apart.

BOX 15-2 ENDO-1, 4- β -GLUCANASES

CELLULOSE IS THE MOST abundant polysaccharide in the world, and although it is produced almost exclusively by plants, enzymes for its breakdown, endo-1,4- β -glucanases, are produced by fungi, bacteria, slime molds, and snails, as well as plants. They are a large group of enzymes that cleave the β -1,4-linkage in chains of cellulose and other polysaccharides possessing a 1,4- β -glucan backbone. Many EGase genes from various organisms have been cloned, and sequence comparisons have helped in their classification into six or more families, with plant EGases belonging to family E.

Microbial E-type EGases possess domains that bind to cellulose, enabling them to degrade crystalline cellulose in plant cell walls. Microbes secrete their cellulases and use the products of degradation as nutrients. Plant EGases, in contrast, lack cellulose-binding domains and are not able to degrade crystalline cellulose. However, they are able to degrade the artificial substrate carboxymethylcellulose, a β -1,4-linked glucan, in the commonly used bioassay for EGase activity. The term "cellulase," therefore, is imprecise for plant EGases, but, nonetheless, is still used.

The primary target of plant EGases are xyloglucans, and perhaps the noncrystalline regions of the cellulose microfibrils, in the cell wall. Like many other proteins, many plant cellulases are synthesized on the rough Endoplasmic reticulum, targeted to the endomembrane system before secretion to the cell wall. Most are nonglycosylated. In the cell wall, they are thought to function in modifying the wall architecture.

Many developmental events in plants involve architectural modification of the cell wall. These events include cell growth in vegetative tissues, fruit growth and enlargement, differentiation of vascular tissues, fruit ripening, and abscission of organs, such as leaves, flowers, and fruits. Based on the pattern of expression and hormonal regulation, plant EGases fall into several groups. One group contains EGases whose mRNA levels are stimulated by auxin. These EGases have been found in rapidly expanding tissues, such as pea epicotyls, tomato hypocotyls, and poplar cell cultures. Another group includes EGases whose expression is induced by exogenous ethylene and retarded by exogenous auxin. In general, EGases found in the ripening fruit, and in the abscission zones of leaves, flowers, and fruits, fall into this category. Still another group of EGase genes do not seem to be induced by auxins, GAs, or ethylene. They encode proteins that are either anchored to the plasma membrane or are integral to it. Not much is known about these EGases, but mutants defective in them indicate a role in cell elongation and in cell plate formation during cytokinesis.

as the name suggests, cleave 1 \rightarrow 3 or 1 \rightarrow 4 bonds in similarly named polymers. Considerable circumstantial evidence shows that XETs and mixed-link glucanases are involved in wall loosening and cell/organ growth. Auxin-induced growth of excised tissues is associated with a high turnover of XGs in dicots and mixed-link glucans in monocots. Mixed-link glucans are synthesized and then degraded during the growth of grass coleoptiles and leaves. Perhaps the best evidence comes from immunological studies. Antibodies prepared against XG and other glucan fragments inhibit auxin-induced growth, and lectins and antibodies that bind matrix glucans reduce wall autolysis and inhibit auxin-induced growth. In maize leaves, the distribution of XETs is highest in the zone of elongation and declines in the zone of maturation and differentiation. Several genes encoding XETs have been

cloned. They belong to a large family, members of which are differentially expressed by a variety of signals, hormones such as auxins and brassinosteroids (see Section II,4.3), and environmental factors, such as touch, wind, and cold temperature. They are expressed in a variety of situations where wall loosening is involved, e.g., stem/root growth, fruit ripening and pulp formation, and production of lateral roots. Thus, there is strong correlative evidence that XETs, and possibly other glucanases, play a role in auxin-mediated growth. The weakness of the theory lies, however, in the fact that it has not yet been demonstrated that XET or glucanase treatment leads to any increase in the plastic extensibility of walls or wall fragments in acidic solutions. Also, the pH optima of these hydrolases are \sim pH 5.5 to 6.0, higher than the actual pH required for wall loosening *in vitro*.

4.1.3.3. Expansins

Expansins were first isolated in 1992 from cucumber hypocotyls by what has come to be known as the “reconstitution assay.” Because boiling of “native” walls causes them to lose extensibility, it was reasoned that if wall proteins from fresh walls, extracted by salt solutions, were added back to boiled walls, extension should be restored. This is indeed what happened, as shown in Fig. 15-27.

Use of the reconstitution assay, combined with protein purification, led to isolation of two proteins from cucumber walls that induced wall extension *in vitro*. They were called expansins. Expansins have no known enzyme activity for XGs or any other substrate, but nonetheless are thought to bring about wall loosening, presumably by cleaving the hydrogen bonds between the hemicelluloses on the one hand and cellulose fibrils on the other (see Fig. 15-26B). The pH optimum of expansin and that of creep is about the same, \sim pH 4.0.

Expansins have been isolated from oat and rice and from members of the grass family as well. This is

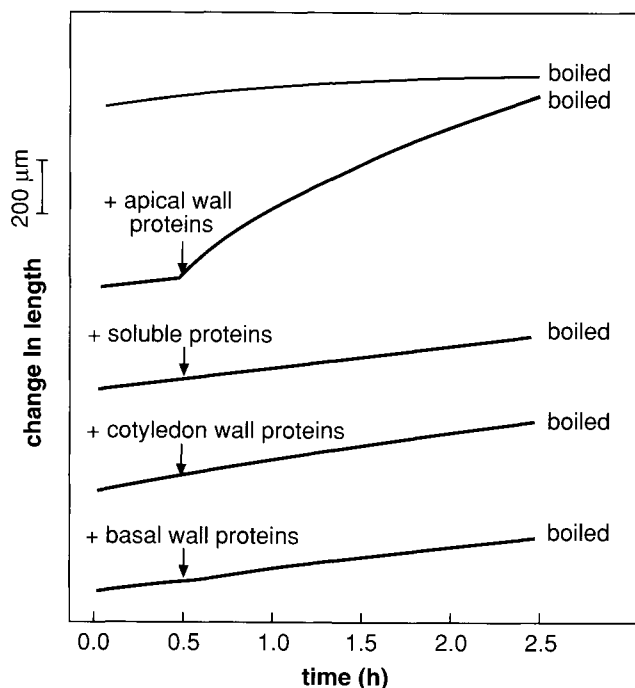


FIGURE 15-27 Extensibility of heat-inactivated cell walls is restored by the addition of wall proteins. Apical 1-cm segments of cucumber hypocotyl were boiled to abolish plastic extensibility (creep). Then salt-extracted (1.0M NaCl) protein fractions from fresh walls were added and their effects on creep measured. Only wall proteins from the apical portion restored creep. Proteins from cotyledon walls, the nongrowing basal part of hypocotyl, or soluble proteins from the cell cytoplasm did not. From McQueen-Mason *et al.* (1992).

particularly important because members of the grass family have the so-called type II walls with a very different chemical composition and wall structure from type I walls seen in dicots and most other monocots (see Chapter 2). Cucumber expansins have been shown by reconstitution assays to restore extension in walls of different species, pea epicotyls, tomato epicotyls, radish hypocotyls, onion and lily leaves, and oat coleoptiles; oat expansin can induce extension in cucumber walls.

As revealed by tissue printing studies and immunogold labeling and electron microscopy, cucumber and rice expansins are present primarily in walls of young-growing organs, not in mature regions. Although expansins are present ubiquitously in growing organs, their pH optimum matches that of the acid growth process ($\text{pH} < 4$) and they can weaken the strength of isolated wall fragments (or paper) *in vitro*; their role in auxin-mediated growth of excised segments or intact stems is still uncertain. Moreover, their biochemical action *in vivo* is unknown.

Expansins are a large family of proteins. Cucumber expansins are still the best characterized. The genes encode \sim 25-kDa mature proteins, which are secreted into the cell wall, but the primary sequence has not revealed any clues as to how the protein might act. cDNAs have been cloned from many other dicots, grasses, and pine and from different tissues (e.g., elongating organs, ripening fruits). Genomic analysis of tomato, tobacco, and *Arabidopsis* reveals that expansins occur as multigene families, but these sequences and their encoded proteins have not been functionally characterized and their specific role in extension growth is unclear.

In summary, while auxins cause acidification of the cell wall and increase its plastic extensibility, the specific roles of expansins and hydrolases, such as EGases and XETs, in wall loosening are not fully understood. It is possible that expansins and wall hydrolases cooperatively modify the hemicellulose–cellulose network during cell growth in young-growing tissues, but more work is needed to demonstrate such cooperation and to clarify their specific contributions.

4.2. GA-Induced Growth

Cells walls are loosened during GA-induced growth, but this wall loosening has not been studied to the same extent as in auxin-induced growth. For instance, it is unclear whether proton extrusion is involved. An acidification of the medium was reported for the GA-induced growth of oat stem segments, but not for lettuce hypocotyl segments. GA treatment of pea epicotyls has been shown to result in an enhanced

XET activity. It has also been reported that the elongation growth of young internodes of deepwater rice is mediated by expansins. Both by immunogold labeling and EM and by tissue printing using Abs against cucumber or rice expansin, expansins were shown to be present in young meristematic, elongating regions, but not in mature regions.

The effect of GAs on elongation growth of whole plant organs or cut segments is unique in that they not only cause an increase in elongation rate of the constituent cells, but they also extend the elongation zone. Thus, in stem segments of deepwater rice, an exogenous application of GA extended the zone of elongation to about twice that in the control (Fig. 15-28). Similar results have been reported for several other systems (e.g., the subapical regions of cucumber hypocotyls, mesocotyl in GA-deficient *d5* maize, elongation zone above the intercalary meristem in leaves of wheat. Commensurate with extension of the elongation zone, GAs have also been reported to extend the zone in which XET activity can be demonstrated.

4.3. Brassinosteroid-Induced Growth

Little is known about the mechanism of BR-induced growth. In intact stems, BRs supplied in high concentrations cause not only elongation, but also splitting of stems (the so-called "brassin" effect). GAs even at

high concentrations do not cause splitting of stems. The cause for splitting is not known, but probably results from an excessive buildup of turgor pressure in the internal tissues. It is unclear whether brassinosteroids bring about acidification of the cell wall, but xyloglucan endotransglycosylase activity has been demonstrated in soybean hypocotyls.

5. HORMONES, MICROTUBULES, AND DIRECTIONALITY OF GROWTH

Several observations suggest that hormones are involved in the orientation of MTs and, thus, indirectly in controlling the directionality of growth. In stem tissues induced to grow rapidly by GA treatment, the orientation of MTs in cortical cells is predominantly transverse. In maize coleoptiles that have stopped elongating, but then are switched to elongate by auxin treatment, there is a rapid change in the orientation of MTs along the OTW of epidermis, from longitudinal to transverse. In ethylene-treated material that shows radial or isodiametric expansion, the orientations of MTs change from transverse to longitudinal or random. ABA treatment inhibits cells from elongating, and cells that had a predominantly transverse

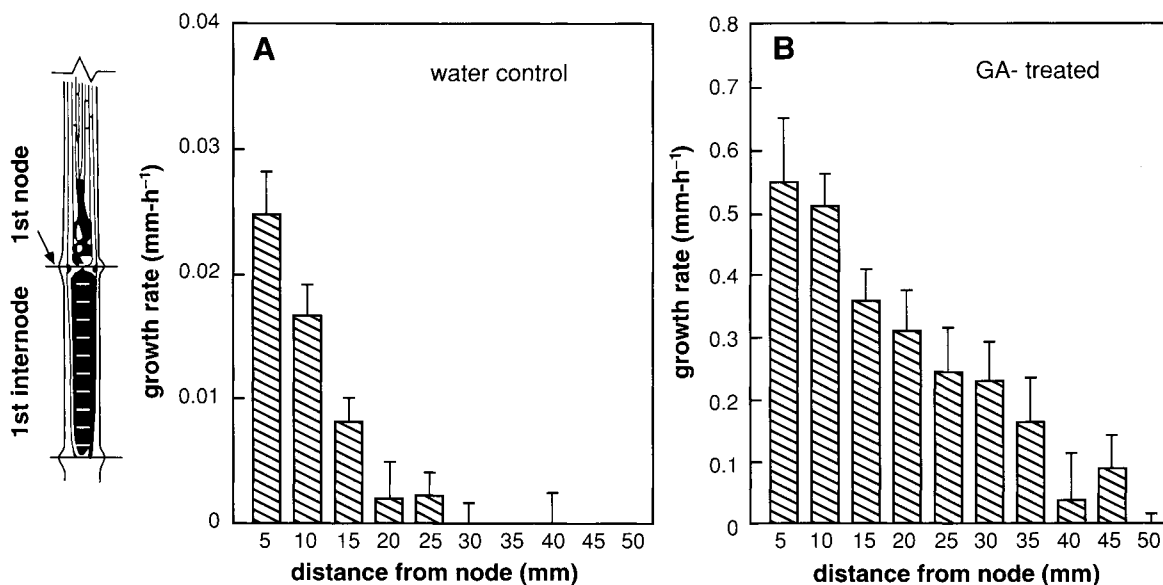


FIGURE 15-28 Distribution of growth along the youngest internode of rice stems. (A) Internodes were marked at 5-mm intervals and incubated in distilled water as control. Growth rates were calculated from the increase in distance between the centers of the marks after 24 h. (B) Internodes were incubated in 50 μ M GA₃ solution for 24 h and then marked at 5-mm intervals. Growth rate was measured after an additional 45 min. Results are averages (\pm SE) from three (A) and two (B) independent experiments with a total of 30 and 29 internodes each. From Sauter *et al.* (1993).

orientation of MTs now show a predominantly longitudinal orientation. These orientations of MTs are, of course, paralleled by orientations of the innermost wall fibrils. The question is do the hormones directly affect the orientations of MTs or do they do so indirectly? The short answer is that we do not really know. At least two observations negate a direct effect of hormones on the orientation of MTs. (i) It is difficult to conceive why some hormones, auxins, GAs, or BRs, should selectively promote a transverse orientation, whereas ethylene or ABA should promote a longitudinal orientation of MTs with respect to the long axis of the cell. (ii) We have seen that epidermal cells during "normal" or hormone-induced elongation growth show a cycling of MTs from longitudinal to transverse orientation against the OTW, whereas cortical (or mesophyll) cells show a transverse orientation. It is difficult to see how both longitudinal and transverse orientations of MTs can occur in epidermal and cortical cells if GA treatment constrains them to be transversely arranged.

The organization of cortical arrays of MTs is believed to be related to asymmetries in the plasma membrane, the microtubule-organizing centers, the microfibrils (clusters of actin filaments), and localized concentrations of ions, such as Ca^{2+} . Hormones could have differential effects on one or more of these factors, but they still need elucidation. It could also be that the hormones simply accelerate growth by wall loosening and/or build up of osmotic pressure, and the pattern and directionality of growth, including the orientation of cortical MTs, are set by some other still unknown factors.

6. GENE EXPRESSION DURING CELL GROWTH

Sustained growth in intact or excised systems requires new synthesis of many proteins, both enzym-

atic and structural, as well as lipids and polysaccharides for membranes and cell walls. Such synthesis for some wall polysaccharides was shown long ago by the incorporation of tritiated glucose in wall polymers for both auxin- and GA-induced growth. That new proteins are synthesized in association with auxin-induced growth has been shown in numerous studies, although the identity of many of these proteins and their specific role in cell growth are still not known (see below). Similar studies for GA-induced growth are still very few.

6.1. Auxin-Induced Genes

Auxin-induced elongation is accompanied by noticeable changes in the profile of proteins that are synthesized: some are downregulated, whereas others are upregulated. This is shown by two-dimensional gels of *in vivo*-synthesized proteins, as well as *in vitro*-translated proteins from mRNAs extracted from auxin-treated vs -untreated controls. These studies, in the early 1980s, utilized, for the most part, intact hypocotyls or hypocotyl segments of soybean (*Glycine max*) treated with 2,4-D, pea (*Pisum sativum*) epicotyl segments treated with IAA, and maize (*Zea mays*) coleoptile segments treated with IAA and led to the isolation of cDNA clones whose transcripts were expressed by auxin treatment. For instance, cDNA clones encoding polypeptides Nos. 1–6 in pea were isolated and named *pIAA1*, *pIAA2*, *pIAA3*, *pIAA4/5* (because of high sequence similarity between the two cDNAs), and *pIAA6*. Some of these cDNAs, isolated to 1987, are shown in Table 15-1. The genes for many of these cDNAs were cloned soon after, and auxin-induced genes are now known from many systems.

mRNAs of these genes are expressed only with physiologically active auxins, e.g., IAA, 1-NAA, and 2,4-D, not with inactive analogs. Also, they are seen only in auxin-treated tissue or elongating tissues; their expression is reduced to low or undetectable levels when auxin is withdrawn, and the addition of auxin

TABLE 15-1 Some Auxin-Regulated Genes and cDNAs

Gene/cDNA	Auxin used	Plant	Earliest detectable change in transcript level	Ref.
<i>pJCW1</i> , <i>pJCW2</i>	2,4-D	<i>Glycine max</i>	15–30 min	Walker and Key (1982)
<i>pGH1</i> , <i>pGH3</i>	2,4-D	<i>G. max</i>	30 min	Hagen <i>et al.</i> (1984)
<i>pIAA 4/5</i> , <i>pIAA6</i>	IAA	<i>Pisum sativum</i>	10–15 min	Theologis <i>et al.</i> (1985)
<i>SAUR</i> (for small auxin upregulated) genes	2,4-D	<i>G. max</i>	2–5 min	McClure and Guilfoyle (1987)

restores their accumulation. In plants transformed with constructs consisting of the promoter sequences of these genes fused to a reporter gene, the reporter gene is expressed by the application of exogenous auxin. Thus, there is abundant evidence that these genes are induced by auxins.

Since the cloning of the first auxin-induced gene in 1989, a wealth of information has accumulated on the structure, regulation, and possible functions of these genes. These topics are covered in Chapter 22.

6.1.2. GA- and BR-Induced Genes

Exogenous application of GA₃ to several elongating systems (e.g., dwarf corn, cucumber hypocotyl, dwarf tomato) has been shown to upregulate the abundance of some and downregulate the abundance of other polypeptides. In some cases, the profile of polypeptides *in vivo* is matched by *in vitro*-translated proteins from RNA extracted from \pm GA-treated materials. However, very little positive information has emerged from studies on gene induction by GAs in elongating systems. Using the procedure of subtractive hybridization, a cDNA clone encoding a protein with homology to a water channel protein (aquaporin) in the vacuolar membrane was identified in dwarf corn (*d1*), and its transcripts were shown to be upregulated by GA₃ treatment. Thus, the protein could be involved in increased water uptake associated with cell elongation. In other studies, the GA₃-induced elongation of oat internode segments was reported to be accompanied by an increase in the transcripts of a tubulin gene.

Brassinosteroid-induced elongation is accompanied by wall loosening, and a gene from soybean encoding a xyloglucan endotransglycosylase has been isolated. It is expressed rapidly after BR treatment, particularly in the inner tissues, phloem, and xylem parenchyma.

Several mutants from *Arabidopsis* are genetic dwarfs, which show a light-grown phenotype when grown in darkness. One class of these de-etiolated (*det*) mutants shows dark green foliage, lack of apical dominance, and even flower bud induction when grown in dark; in light, plants show dwarfism and delayed senescence. These mutants typically are deficient in endogenous BRs, are insensitive to applied BRs, and are discussed in greater detail in Chapters 24 and 26. Here it is relevant to point out that the *det3* mutant shows dwarfism because of reduced cell elongation in hypocotyl, petioles, and inflorescence stems (but not in leaves). The *DET3* gene encodes the C subunit of a vacuolar H⁺-ATPase. These ATPases are multimeric complexes, which secrete protons into the cytosol. The proton gradient so created is used to drive the uptake

of other ions and solutes into the vacuole. The osmotic potential of the vacuole is thus raised and, in turn, causes influx of water which is necessary for cell growth.

7. ASYMMETRIC GROWTH

In addition to the more or less symmetrical elongation of axial organs that has been considered so far, there are many instances of asymmetric growth of axial organs. The well-known phenomena of bending of shoots and roots in response to light (phototropism) or gravity (gravitropism) involves asymmetric growth, which is mediated in part by auxin. It is covered in Chapter 27. Other examples of asymmetric growth include apical hook formation/maintenance in dicot seedlings, coiling of tendrils, stems or leaves, and nastic growth. Here we consider apical hook formation and opening, a well-investigated but still little understood response. Coiling of tendrils and epinasty are mentioned very briefly.

7.1. Apical Hook Formation and Opening

The apical hook formation and opening involves asymmetric growth on two sides of the hypocotyl/epicotyl and is regulated by a complex interaction between auxin, ethylene, and light. Several observations indicate the involvement of auxin. If radiolabeled IAA is applied to the shoot apex of bean seedlings, the radiolabel moves basipetally and shows a differential distribution in the hook region. If such transport is inhibited by application of naphthylphthalamic acid (NPA) or triiodobenzoic acid (TIBA), a hook is not formed. Not only an inhibition of polar transport, but also an oversupply of auxin results in a hookless phenotype (e.g., *Arabidopsis sur1/hls3/alf1* mutant that overproduces auxin, see Chapter 6). Also, *Arabidopsis* mutants defective in auxin sensitivity or signaling (e.g., *axr1-12* or *nph4*, see Chapters 22 and 27) are hookless when grown in air. These data suggest that auxin distribution and/or homeostasis in the seedling plays a role in hook formation and/or maintenance.

The involvement of ethylene is also indicated by several observations. Seedlings grown in air form a modest hook, which is exaggerated in the presence of ethylene. Also, seedlings grown in darkness and supplied ethylene form a tighter apical hook (see Fig. 11-1, Chapter 11). Inhibitors of ethylene synthesis (e.g., aminoethoxyvinylglycine) or action (e.g., 2,5-norbornadiene), supplied to intact seedlings, give a much

reduced apical hook. In conformity with the above result, *Arabidopsis* mutants that are insensitive to ethylene (e.g., *etr11*, *ein2*) show a much reduced hook. By contrast, ethylene overproducing or constitutive response mutants (e.g., *eto1* or *ctr1*) show an exaggerated hook (for ethylene-response mutants, see Chapter 21). Interestingly, *eto1* and *ctr1* mutants treated with auxin transport inhibitors, as in the wild-type, fail to form an apical hook, which means that ethylene action in hook formation/maintenance is dependent on an intact auxin balance.

The *hookless1* (*hls1*) mutant of *Arabidopsis* is an organ-specific mutant, which shows ethylene-insensitivity in the apical hook region, but not in the hypocotyl or the root (see Chapter 21). Mutants carrying severe alleles of *hls1* fail to form an apical hook when grown in darkness and supplied ethylene, although mutants carrying milder alleles respond normally. The *HLS1* gene encodes a protein with similarity to *N*-acetyltransferases found in bacteria, yeast, and mammals, and although it plays a role in hook formation or maintenance, its precise role is not clear. *HLS1* transcripts are expressed throughout the hook region, and their incidence is increased by ethylene treatment; furthermore, overexpression of *HLS1* in transgenic plants causes a hook to be formed constitutively. Treatment of wild type (*HLS1*) seedlings with auxin transport inhibitors, NPA or TIBA, decreases the incidence of *HLS1* mRNA. Two *HOOKLESS* genes have also been cloned in pea (*Pisum sativum*), and one of them is expressed in response to ethylene.

Measurements of cell length indicate that cells on the inner, concave side of the hook are about half the length of cells on the outer convex side. It is generally thought that ethylene produced by cells on one side of the epicotyl/hypocotyl (the future concave side of the hook) inhibits cell elongation causing the curve to develop and to be maintained. Accordingly, efforts have been made to determine endogenous ethylene levels in cells on the inner and outer sides of the hook. *In situ* measurements of ethylene levels are difficult, but the relative amounts of ACC (the immediate precursor of ethylene), and transcripts of ACC oxidase (the enzyme that oxidizes ACC to yield ethylene, see Chapter 11) have been determined in pea, bean, and *Arabidopsis*. Unfortunately, the results to date do not yield a clear picture. In pea epicotyls, as shown by *in situ* hybridization, cells on the inner side accumulate more transcripts of an isoform of ACC oxidase (coded by *PsACO1*), than do cells on the outer side (Fig. 15-29). The distribution of ACO enzyme activity follows the same pattern. By contrast, in bean a higher concentration of ACC is reported in cells on the outer convex side of the hook than on the inner side.

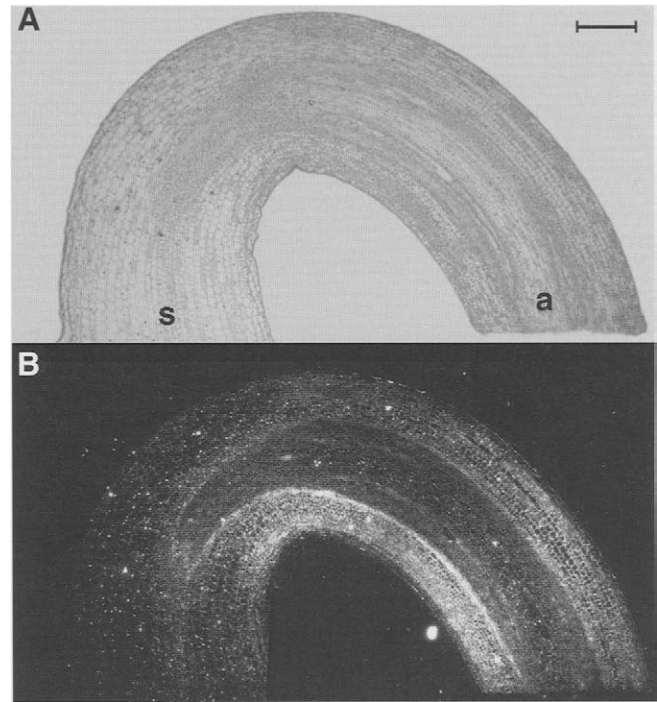


FIGURE 15-29 Localization of *Ps-ACO1* mRNA in the apical hook of etiolated pea (*Pisum sativum*) seedlings. Apical hooks were isolated from etiolated 5- to 6-day-old air-grown seedlings. Hybridization was performed with a ^{35}S -labeled antisense strand of *Ps-ACO1*. The apex (a) is toward the right and the straight stem (s) is to the left. (A) Bright-field image and (B) dark-field image. Bar: 0.45 mm. From Peck *et al.* (1998).

Similarly, in *Arabidopsis* higher transcript levels of an isoform of ACC oxidase (*AtACO2*) are recorded on the outer side than on the inner side. Obviously more work is needed to reconcile these data.

Until recently, it has been generally accepted that hook formation involves only differential cell growth. Recent work on *Arabidopsis* wild-type plants and mutants which lack a hook indicates that not only differential cell growth, but also differential cell divisions are involved in hook formation. Also, that the apical hook formation and maintenance proceeds in at least two stages. In the first stage a modest hook is formed and maintained; this stage is either not regulated or negatively regulated by ethylene. The second stage (between 60–72 h after germination), is sensitive to ethylene and seedlings supplied ethylene at this stage form a tighter hook. Mutants such as *hls1* form a modest hook, but fail to maintain it in the first stage and, as a result, show ethylene-insensitivity. It is not known whether the first stage is regulated by auxin. Also unknown is the nature of the interaction between auxin and ethylene. Auxin at high concentrations is known to induce ethylene production, and ethylene is

known to inhibit polar transport of IAA, but how the two hormones interact to regulate hook formation and maintenance is not clear.

On emergence from soil, or if dark-grown seedlings are exposed to red or white light, the hook straightens because now the inner side grows faster than the outer side. Apical hook opening in nature is a phytochrome-mediated response, and Arabidopsis mutants that show a constitutive de-etiolated phenotype in dark also show an open hook (e.g., *cop2* mutant) (see Chapter 26). How light brings about the change in growth pattern or interacts with auxin and/or ethylene is unknown. Cytokinin overproducing mutants (e.g., *amp1*) also show an open hook (and a de-etiolated phenotype) in dark-grown seedlings. That response has been attributed to ethylene production by cytokinin.

A different version of apical hook is seen in ferns. In ferns, the young leaves show coiling at the tip (circinnate vernation) and the young leaves are called fiddleheads. In some ferns, leaves are produced underground; they have to push their way up through the soil and the coiled leaf tip may aid in the process. As the leaf ages, or upon emergence from soil, the leaf tip unfolds. Little is known about the hormonal or environmental regulation of coiling and uncoiling of leaf tips in ferns.

7.2. Coiling Growth

An extreme version of asymmetric growth is seen in many climbing plants (lianas or vines) that coil around a support. Coiling may be accomplished by the sub-apical parts of the shoot tip, petioles, leaf tips, or special stem or leaf modifications known as tendrils [e.g., pea (*Pisum sativum*), grape (*Vitis vinifera*), *Clematis* sp.]. The mechanism of coiling growth is not understood, although it is probably accomplished by growth inhibition on the inner side of the coil. As mentioned in Chapter 12, the coiling of tendrils is induced by touch (it is a thigmotropic response), by exposure of plants to methyl ester of jasmonic acid (JA), and, even more potently, by the methyl ester of 12-oxophytodienoic acid (12-O-PDA), an intermediate in the synthesis of JA. It has been suggested that touch induces the synthesis of methyl 12-O-PDA, which in turn elicits IAA accumulation in the organ. How IAA brings about differential growth on two sides is not clear, but it does not seem to involve ethylene because inhibition of ethylene production by ethylene synthesis inhibitors does not affect coiling induced by auxin. Whether ethylene induces accumulation of jasmonates is not known.

7.3. Epinasty

Exposure of plants or plant parts to ethylene causes drooping of leaves and flowers, a phenomenon known as epinasty. Epinastic curvature results from unequal (or asymmetric) growth on the upper and lower sides of petioles or peduncles. The upper (adaxial) side grows at a faster rate than the lower (abaxial) side resulting in a downward curvature of the organ (see Fig. 11-2, Chapter 11).

8. SUMMARY OF HORMONE-INDUCED CELL EXPANSION

There is abundant evidence from both hormone synthesis mutants and application of hormones to intact plants and cut segments that hormones induce cell expansion. Such induction is seen only in systems that are developmentally or environmentally programmed to grow. The directionality of growth in multicellular axial organs is determined primarily by the orientation of MTs and innermost cellulose fibrils in cortical (or mesophyll) cells, but the rate of such growth is regulated by cross-lamellate walls, especially the OTW, of epidermal cells. Whereas auxins are believed not to affect the osmotic pressure of growing cells, gibberellins, in some cases, have been reported to affect osmotic pressure by the hydrolysis of stored products. Auxins, GAs, and BRs are all believed to cause wall loosening, although the phenomenon is better documented for auxins than for GAs or BRs. Auxin-induced growth is accompanied by an acidification of the cell wall by activation of a proton ATPase at the plasma membrane. Expansins in the young cell walls are activated with the acidic pH and are believed to break the hydrogen bonds between hemicelluloses and cellulose fibrils, thus allowing cell expansion to occur under the motive force of water uptake. Various wall hydrolases, especially XETs, are induced by auxins; they may also play a role in cell growth by breaking hemicellulose chains and causing their rearrangement. When cell expansion slows down or ceases, hydrogen bonds are formed spontaneously between cellulose fibrils and hemicelluloses. A similar chain of events probably occurs in GA- and BR-mediated growth, but the acidification of the wall is uncertain. During the period of expansion, the directionality of expansion is controlled by the orientation of microtubules and innermost cellulose fibrils in the cortical cells, whereas the overall rate of expansion is regulated by epidermal cells. Whether hormones regulate the orientation of microtubules and, thereby,

innermost wall fibrils and directionality of growth is not clear, but a direct effect of hormones on assembly/orientation of microtubules seems unlikely. A large number of genes are expressed during auxin-induced growth, but the functions of the encoded proteins in relation to cell growth remain unknown. The number of genes that are known to be induced during gibberellin- or brassinosteroid-induced growth is still too small, but some of them have a known function either in water relations of the cell or in wall loosening.

Several plant hormones affect cell expansion. Among these auxins, GAs and BRs are involved in elongation growth, whereas ethylene promotes radial expansion (cytokinins are known to promote radial expansion also, but that effect may be due to CK-induced ethylene production). Auxins, ethylene, and jasmonates are also involved in asymmetric growth on two sides of an axial organ. Assuming that the mechanical basis for cell expansion is the same, i.e., wall loosening accompanied by enhanced turgor pressure, synthesis of wall polysaccharides, and cytoskeletal rearrangements, a question of fundamental importance is how these different hormones mediate the same or similar effects by different signaling pathways. It is possible that subtle differences exist between the characteristics of elongation growth mediated by GAs and BRs and between GAs and BRs on the one hand and IAA on the other and, in case of asymmetric growth, among auxins, ethylene, and jasmonates. An elucidation of these questions would be a major undertaking for future research.

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16

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1. PLANTS ARE EXPOSED TO STRESSES OF VARIOUS KINDS

Stress is defined as an external factor that exerts a disadvantageous influence on photosynthesis, productivity, general vigor, or overall growth of a plant. Plants, being rooted, are subject to various types of stresses caused by both biotic and abiotic factors. Abiotic factors include water deficit (drought), increased salt concentration (salinity), extremes of temperature, sudden changes in temperature, i.e., cold or heat shock, oxygen deficiency in soil due to flooding, presence of reactive oxygen radicals, and exposure to high light intensity in the photosynthetically active part of the spectrum, UV-A and -B. Stressed plants are also susceptible to attack by pests and pathogens. Plants have evolved to perceive these stresses and to cope with them using some evolutionarily conservative mechanisms, and some that are specific to plants. This chapter concentrates mainly on the responses

of plants to three types of abiotic stresses—drought, salinity, and freezing—because each one has some component(s) that is regulated by ABA as well. Other abiotic stresses, such as high irradiance or increased UV, or stresses caused by biotic factors, such as pests and pathogens, may have an ABA-regulated component, but are not covered in this chapter.

1.1. Different Types of Stresses Elicit Different as Well as Some Shared Responses

Water deficit, caused by a combination of low soil moisture level and water loss by transpiration, is one of the most common stresses experienced by plants. Even plants growing in areas of abundant annual rainfall and moderate temperatures (mesophytic climate) are exposed periodically to water deficit, e.g., in the middle of a hot summer day or during a succession of warm, dry days/nights. Water deficit also results from the salinity of soil or by freezing temperatures. Salinity lowers the free water content (water potential, Ψ) of the soil, thus making it less available to roots and causing what is known as physiological drought. Freezing also lowers the free water potential. Ice formation usually occurs in the extracellular space or apoplast (intracellular ice formation is usually lethal) and results in loss of intracellular water to the apoplast, causing dehydrative damage. Drought, salinity, and freezing temperature are separate factors that elicit different responses from plants, but they also have one thing in common: they lead to dehydration of cells and tissues and attendant damage to membranes

and macromolecules. Because of this common feature, some of the responses to drought, salinity, and freezing are shared responses. Moreover, a response to one stress often protects plants against other stresses with which it shares a common feature—a phenomenon known as **cross protection**. For example, winter wheat, rye, or spinach plants subjected to water stress for a few days can tolerate much lower freezing temperatures than those not stressed. Similar cross protections are known between heat shock and salt tolerance, or tolerance to heavy metals or to pathogens.

1.2. Mesophytes and Plants Living in Extreme Habitats

Plants living in extreme habitats of desert (xerophytes) or salt marshes (halophytes), and the special group of plants known as “resurrection” plants (see Box 16-1), have evolved special mechanisms to cope with long periods of drought or salinity. In contrast, mesophytes, have no such special mechanisms; nonetheless, they also are exposed periodically to drought conditions. The most visible manifestation of water deficit in mesophytes is the phenomena of wilting—drooping of leaves and aboveground parts—which results from loss of turgor by cells/tissues. Under continued water deficit, leaves may also be shed. At the cellular level, dehydration causes many other problems, including cell volume shrinkage, increased solute concentration in the cell, inhibition of enzyme activity, changes in membrane lipids, and build up of free radicals and attendant damage to membranes.

BOX 16-1 LIFE IN EXTREME HABITATS

PLANTS LIVING IN EXTREME habitats of desert (xerophytes) have special adaptations to cope with long spells of hot, dry weather. Annuals germinate, grow, flower, and set seed in the short 2–3 months of favorable climate and spend the rest of the unfavorable year as seeds. They avoid the problem of drought. The perennials have either extremely long roots, which penetrate deep into the soil to reach the low water table (e.g., the legume mesquite, *Prosopis* sp.), or else have abundant storage capacity for water, which they collect during the short rainy season (e.g., succulents, such as *Saguaro*). At the same time they cut down on transpirational loss by keeping their stomata closed during the day time and reducing surface area by replacing leaves with thorns.

Halophytes live in salt marshes and are exposed to excessive salt as well as physiological drought. The ice plant (*Mesembryanthemum crystallinum*) is a succulent that grows along sandy beaches of the California coast and is exposed to extreme salinity. These plants sequester salt (NaCl) in the cell vacuole and deposit it in a gradient along their axis, with the highest concentrations ($\sim 1.0\text{ M}$ or more) in the younger growing parts. In addition, they deposit a sugar alcohol, D-pinitol, in a parallel fashion in the cytoplasm. The

deposition of salt and pinitol, in vacuoles and cytoplasm, respectively, lowers the free water potential and enables the young growing parts of these plants to reduce loss of water to the surrounding medium. They also have special "salt" glands on leaves, which concentrate salt and exude it to the outside where it may dry, giving the appearance of ice; hence, the name ice plant.

Many species living in deserts or salt marshes also show C_4 photosynthesis, C_3/C_4 intermediate photosynthesis, and Crassulacean acid metabolism (CAM). Plants showing CAM keep their stomata closed during the day. Stomata are opened at night when it is more humid and the air temperature is cooler. CO_2 is taken up at night and is assimilated into oxaloacetate by the enzyme phosphoenolpyruvate carboxylase (PPC) and finally into malate. Malate is stored in vacuoles from where it is mobilized during the daytime, providing CO_2 for the main carbon-fixing enzyme, RUBISCO. In the citric acid cycle (Krebs cycle) in mitochondria, CO_2 is also taken up by phosphoenolpyruvate to provide oxaloacetate, a reaction catalyzed by the same enzyme PPC. CAM plants have at least two isoforms of the enzyme. One is expressed constitutively at low abundance and serves the housekeeping function of supplying C_4 intermediates to the citric acid cycle. The other is the CAM-specific isoform and is expressed in abundance when plants are under stress. The two are encoded by separate genes. C_4 plants show a modified CAM in that the malate is cycled between mesophyll and bundle sheath cells. It is formed from oxaloacetate in the mesophyll cells and is transferred to bundle sheath cells where it releases CO_2 for RUBISCO. Phosphoenolpyruvate (PEP) formed from malate when CO_2 is released is transferred back to mesophyll cells.

"Resurrection" plants are a special case. They are a small group of about 100 angiosperms termed poikilohydric or "resurrection" plants, which have the unique property that they can lose water to the point of almost complete dryness; they look "dead" for all practical purposes, yet are resurrected on rewatering. One of the best studied resurrection plants is *Craterostigma plantagineum* from South Africa (Fig. 16-1). *Craterostigma* plants need constant watering to grow, but if deprived of water, they can stay alive in a state of dryness (about 2–3% relative moisture content) for indefinite periods. However, when watered, they resume their full physiological and growth functions within 12–15 h. The alternate cycles of drying and hydration can be repeated many times in the laboratory or greenhouse, provided a sufficient time for growth, photosynthesis, and recuperation of resources is allowed during the hydration period.

The ability to survive in a desiccated state is not unique to angiosperms. Some ferns and lycopods (e.g., *Polypodium virginianum*, *Selaginella lepidophylla*), a moss *Tortula ruralis*, lichens, and several intertidal algae, such as the red alga *Porphyra*, and green alga *Ulva*, show the same properties.



FIGURE 16-1 *Craterostigma plantagineum* plants. (A) Fully turgid plant. (B) Desiccated plant (unwatered for 7 days). (C) Plant rehydrated for 6 h. With permission from Bartels *et al.* (1990), ©1990 Springer-Verlag.

1.3. How Do Mesophytes Respond to Water Deficit?

Mesophytes, which include the majority of plants, trees, shrubs, and most crop plants, respond to water deficit by a closure of stomata to cut down on a loss of water through transpiration. Stomatal response can be very rapid; depending on the species, stomata can close within minutes of water stress being applied, before leaf cells have lost their turgidity. Stomatal closure is usually accompanied by the inhibition of

photosynthesis and cessation of shoot growth, although root growth may continue or may even be enhanced. Water deficit also leads to accumulation or synthesis of low molecular weight compounds (osmolytes), as well as synthesis of new proteins thought to protect membrane and macromolecular surfaces and to combat against a buildup of oxidants and free radicals. When relief is provided by rehydration, stomata open again, photosynthesis and shoot growth resume, and the production of osmolytes and protective proteins stops. Many plants, such as grasses, can survive

long periods of drought in this state of semidesiccation, but for others, a persistent drought can be lethal.

Many of the responses just given are common to plants living in desert and salt marshes and in resurrection plants. They are also seen during seed development in angiosperms and gymnosperms, although, in contrast to the desiccation tolerance by the vegetative parts, which is induced environmentally, seeds represent a dehydrated state that is regulated developmentally. A knowledge of biochemical and molecular mechanisms by which plants living in extreme habitats tolerate drought or cope with excess salt, or by which seeds desiccate, is useful in understanding stress tolerance by mesophytic plants.

1.4. Scope of the Chapter

This chapter deals mainly with stress tolerance by mesophytes. Desiccation of seeds is covered in Chapter 18. We first consider the evidence that abscisic acid (ABA) is involved in stress tolerance, followed by the role of ABA in the regulation of physiological processes in the shoot and in freezing tolerance. These sections are followed by the accumulation of low molecular weight osmolytes and stress-induced gene expression, including possible functions of some stress-related proteins. A final section deals with some efforts to engineer stress tolerance in some crop plants.

2. ABSCISIC ACID (ABA) PLAYS A CRUCIAL ROLE IN MEDIATING MANY RESPONSES TO ENVIRONMENTAL STRESS

Irrespective of whether desiccation is regulated developmentally as during seed maturation or whether it is enforced environmentally, the mechanism of plant response seems to be similar and involves the hormone abscisic acid. ABA plays a crucial, perhaps a central, role in mediating some of these responses, although other responses seem to be regulated independently.

That ABA is involved in countering the effects of desiccation is indicated by several lines of evidence.

2.1. Water Stress Causes a Rapid but Transient Accumulation in Endogenous ABA

As explained in Chapter 10, dehydration of intact plants or detached organs causes a rapid increase in endogenous ABA by an increase in the rate of ABA synthesis. Such a rise in ABA levels is brought about

by all factors that result in cellular dehydration: drought, increased salinity, exposure to chilling temperatures, or placement in a solution of high osmolarity (Fig. 16-2). The accumulation of ABA is transient. Rehydration of wilted plant parts leads to a rapid decline in the ABA content. However, more importantly, even if water deficit continues, after a certain time, which varies with plant and organ (usually 8 to 24 h, but longer times are known), the levels of ABA decline. The decline is due to the stoppage of synthesis and increased conversion to metabolites, such as phaseic acid and dihydrophaseic acid. The transient nature of the increase in ABA levels suggests that ABA acts as a signal to bring about certain responses. Some of these responses are fast (e.g., stomatal closure), whereas others take longer and involve gene expression. After the responses are affected, the continued presence of ABA may not be required.

2.2. ABA Can Mimic Some Effects of Drought Stress

Application of exogenous ABA has been reported in many cases to induce cessation of bud and leaf growth and inhibit photosynthesis (but see Section 3.3). In detached, but nonstressed, leaves that are supplied

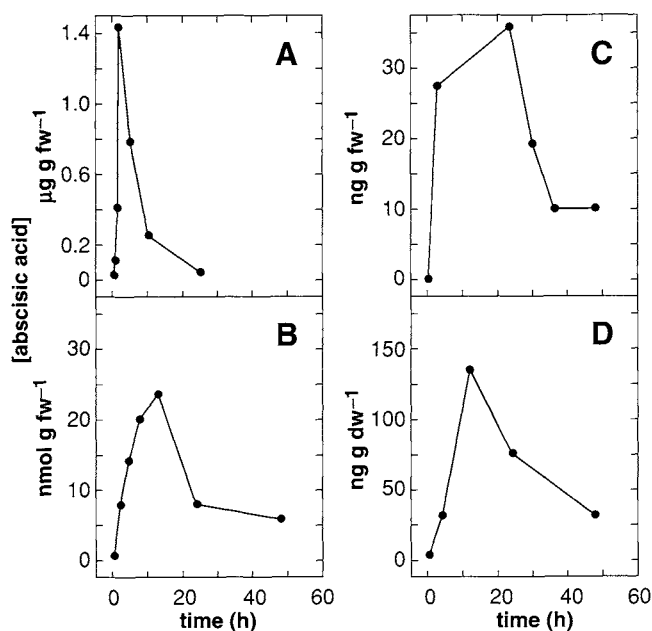


FIGURE 16-2 Examples of stress-induced ABA transients. (A) Detached wilted leaves of rice. (B) Roots of salt-stressed rice seedlings. (C) Polyethylene glycol (PEG)-treated submerged shoots of *Hippuris*, an aquatic plant. PEG acts as an osmolyte and desiccant. (D) Cold-acclimated wheat shoots. Data compiled from various authors by Cowan *et al.* (1997).

ABA, it induces closure of stomata. (It should be noted that stomatal pore behavior is regulated by multiple factors—light, humidity, and CO_2 concentration—and ABA is only one of the factors involved.) Stomatal closing is not an all-or-nothing phenomenon—stomata usually open (or close) partially. Stomatal conductance is the term used to denote the degree of stomatal pore opening; the greater the opening; the more the conductance of water vapor and CO_2 through stomata. Stomatal conductance, or its reverse, stomatal resistance, is measured using a porometer. Figure 16-3 shows that ABA fed to detached cherry leaves via petioles causes a decline in stomatal conductance.

Cessation of growth and stomatal closing are complex responses, which are governed by numerous factors; hence, these correlations are not always obvious with whole plants or under field conditions. In comparison, expression of a specific gene (or its encoded protein), although a complex response, is simpler to measure.

2.3. Dehydration Causes Synthesis of New Proteins That Are Also Induced by ABA

A comparison of two-dimensional (2-D) polyacrylamide gel electrophoresis (PAGE) of proteins extracted from unstressed shoots and those that have been allowed to wilt shows that while many proteins are downregulated under water stress, many new proteins are synthesized (Fig. 16-4). These results are confirmed by 2-D PAGE of *in vitro*-translated mRNAs extracted from stressed and unstressed leaves.

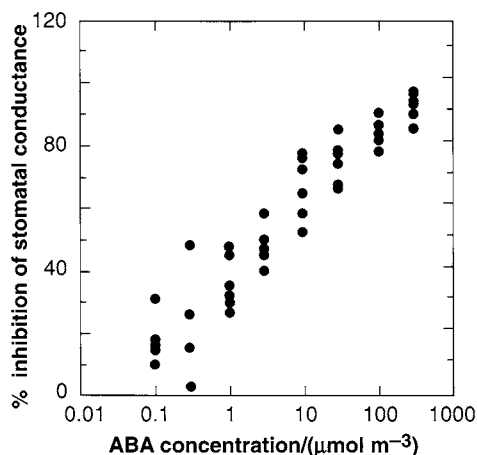


FIGURE 16-3 Inhibition of stomatal conductance of cherry leaves by ABA. A range of ABA solutions were fed continuously to the cut petioles until the closing response was complete. Each point represents an individual leaf. Data show clearly that as the concentration of fed ABA increased, stomatal pores were closed to a greater and greater extent. From Gowing *et al.* (1993).

Together, these data suggest that water stress causes downregulation of expression of many genes and a concomitant upregulation of many other genes. Many of these genes induced by dehydration or desiccation have been cloned from a variety of plants, including tomato, pea, maize, wheat, barley, and the resurrection plant, *Craterostigma plantagineum*. What is remarkable is that many of the same genes, but not all, are also induced by an exogenous application of ABA to unstressed plants. The converse is also true. Some of the genes that are upregulated by ABA are not upregulated by water stress.

The correspondence between genes that are induced by water stress, as well as exogenous ABA, suggests that the induction of many genes under water stress is

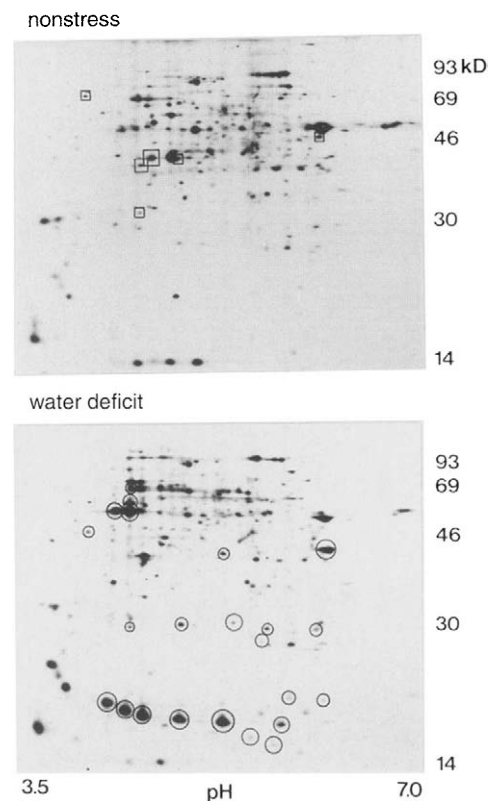


FIGURE 16-4 Changes in polypeptide synthesis in response to drought stress. Tomato leaves were kept in water (nonstress) or were drought stressed by wilting to 85% of their original fresh weight (water deficit). Leaflets were detached and were supplied L-[^{35}S] methionine through the petiole for 2 h. Subsequently, proteins were extracted and separated by 2-dimensional PAGE. This protocol identifies only those proteins whose synthesis occurs during the 2 h incubation period. The synthesis of the majority of polypeptides is not affected by drought stress. However, the concentration of ~22 individual polypeptides was increased in response to drought stress. The more prominently radiolabeled polypeptides are circled. Polypeptides whose synthesis was decreased during drought stress are indicated by squares. From Bray (1990).

mediated by endogenous ABA. Support for this suggestion comes from the use of ABA synthesis mutants.

2.4. ABA Synthesis Mutants Are Unable to Cope with Water Stress

In Chapter 10, several ABA synthesis mutants were noted that are defective in the terminal step of ABA biosynthesis: the oxidation of ABA aldehyde to ABA (e.g., *flacca* and *sitiens* of tomato, *aba3* of *Arabidopsis*, see Fig. 10-8). These mutants are deficient in endogenous ABA. Under water stress, they are unable to close their stomata and are prone to wilting. They are also unable to synthesize many ABA-regulated proteins. However, if exogenous ABA is supplied to the plants, the stomata close normally, and ABA-regulated proteins are synthesized. Figure 16-5 shows the expression of

three mRNAs expressed under water stress in the leaves of the wild-type tomato, but not in the *flacca* mutant. The same mRNAs are also expressed in the wild-type tomato, as well as in the *flacca* mutant when the leaves are supplied with exogenous ABA.

The evidence just given points to ABA playing an important regulatory role in mediating the expression of some genes related to water deficit. Other genes are regulated independently of ABA, as is shown by studies utilizing ABA response mutants. This topic is covered elsewhere in this chapter. However, it can be indicated here that ABA response mutants, like ABA synthesis mutants, fail to close their stomata and are prone to wilting under water stress, but, unlike synthesis mutants, are not rescued by a supply of exogenous ABA.

3. ABA AND REGULATION OF PHYSIOLOGICAL PROCESSES IN SHOOTS

Drying of soil causes an increase in ABA concentration both in shoots and in roots, and most of this increase is by *de novo* synthesis of ABA. The question arises whether it is the ABA synthesized in the shoots or that in the roots of an intact plant that plays the major role in triggering events that occur in the leaf, i.e., stomatal closure, cessation of growth, and cessation of photosynthesis? In order to answer this question, we must first ask how plants sense drying and where this signal is perceived.

3.1. How Do Plants Sense Drying?

Dehydration results in a decline in water potential, an increase in solute concentration, and a decline in turgor pressure of the cells. Among these factors, loss of turgor is associated most closely with ABA synthesis (see Section 7.1 in Chapter 10), although how the loss of turgor, or related perturbation in the plasma membrane, is translated into activation of ABA biosynthesis is unknown. In intact, rooted plants, it is also a matter of debate whether the signal is first perceived in the leaves or in the roots. Because leaves are the place where most transpirational loss occurs, it has been suggested that a drop in water potential in the leaf, which would be aided by a declining supply of water from the roots, is the signal for stomatal closure. However, an alternate view has also been proposed: that the soil-water status, not the level of water in the shoot, is the signal for stomatal closure and arrest of shoot growth. According to the latter hypothesis, roots sense the drying of soil and transmit that signal to the

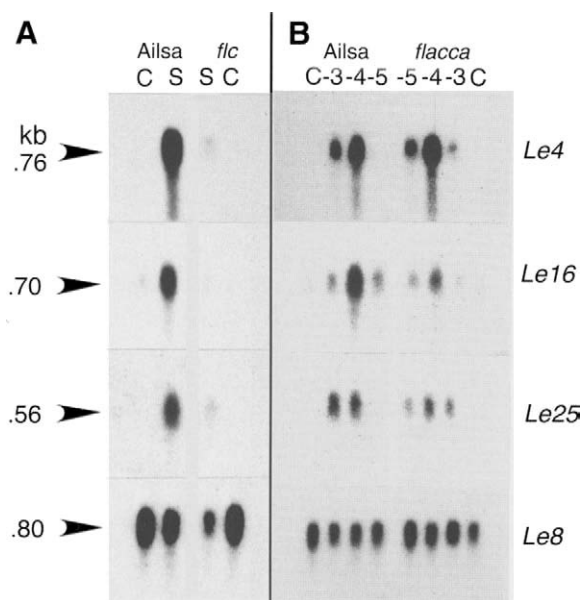


FIGURE 16-5 Northern blots showing the expression of three mRNAs in the wild-type tomato (Ailsa Craig) and the *flacca* mutant. (A) Effect of water stress on the accumulation of transcripts of three cDNA clones: *pLE4*, *pLE16*, and *pLE25*. Note that transcripts accumulate in leaves of the wild-type plants under stress (S), but not in unstressed leaves (C). They are also not expressed in leaves of the *flacca* mutant, whether under stress or not. (B) Effect of exogenous ABA on the expression of the same transcripts in unstressed plants. Detached leaves were treated with water (control, C) or with ABA for 6 h (3, 4, 5). Three concentrations of ABA were used. Optimal results were obtained with 10^{-4} M ABA (4); the 10^{-5} M concentration (5) was too low and 10^{-3} M (3) was inhibitory. The transcripts are not expressed in the controls, but are expressed under the influence of ABA both in the wild type and in the *flacca* mutant. Transcripts of a fourth clone, *pLE8*, are present in both wild-type and *flacca* mutant leaves under nonstressed conditions; the clone was included as an internal control. With permission from Cohen and Bray (1990), ©1990 Springer-Verlag.

above ground shoot—stem and leaves—*via* a chemical signal, thought to be ABA. Several lines of evidence support this assumption.

3.1.1. Kinetics of Stomatal Pore Closure

If one measures the kinetics of stomatal pore closure under controlled dehydration of soil, it can be shown that pore closure is related more closely to soil water status than to leaf water status, and indeed the pores close before there is any significant drop in the leaf water potential.

3.1.2. Correlation between Root ABA Synthesis and Xylem Sap ABA

Many studies utilizing herbaceous plants, conifers, and tropical trees have demonstrated that, as a result of drying of soil, the ABA content of roots rises and that this rise is correlated with a rise in the ABA content of xylem sap. The ABA concentration in xylem sap of well-watered herbaceous plants, such as maize and sunflower, is about 10 nM ; this concentration can rise as much as 100-fold by mild drying of the soil, drying that does not significantly affect the leaf water potential. Figure 16-6 shows enhanced accumulation of ABA in maize roots subjected to air drying. It also shows that in both maize and a tropical tree, *Leucaena leucocephala*, there is almost a linear correlation between root ABA content and ABA concentration of the xylem sap, more so in maize than in *Leucaena*.

Soil that is compact or has been compacted artificially has a higher density, lower porosity, and much less water content than uncompacted soil. Plants grown in these soils also show an increase in xylem sap ABA.

3.1.3. Stomatal Conductance Is Correlated to Xylem Sap ABA

Studies correlating stomatal conductance, leaf water potential, and concentration of ABA in the xylem sap, as well as in the total leaf, have shown that stomatal pore closing is correlated most directly with the concentration of ABA in xylem sap, and not with the water potential or the concentration of ABA in the leaf (Fig. 16-7). The root water potential and xylem ABA concentration seem to have a direct relationship to stomatal behavior, not shoot water potential.

Despite the evidence just given, the relationship between root and xylem sap ABA and stomatal pore closure is not as clear as one would like. The rise in xylem sap ABA is not always very high on drying of the soil, and, in field-grown plants, the correlation between xylem sap ABA and stomatal conductance is often not seen. The situation in whole plants is further complicated because an enhanced accumulation of ABA in xylem sap may have a component that is derived from ABA synthesized in the shoot, which is also under water stress. Also, ABA translocated basipetally from the shoot in the phloem may be transferred, *via* transfer points in the root or shoot, to the xylem stream.

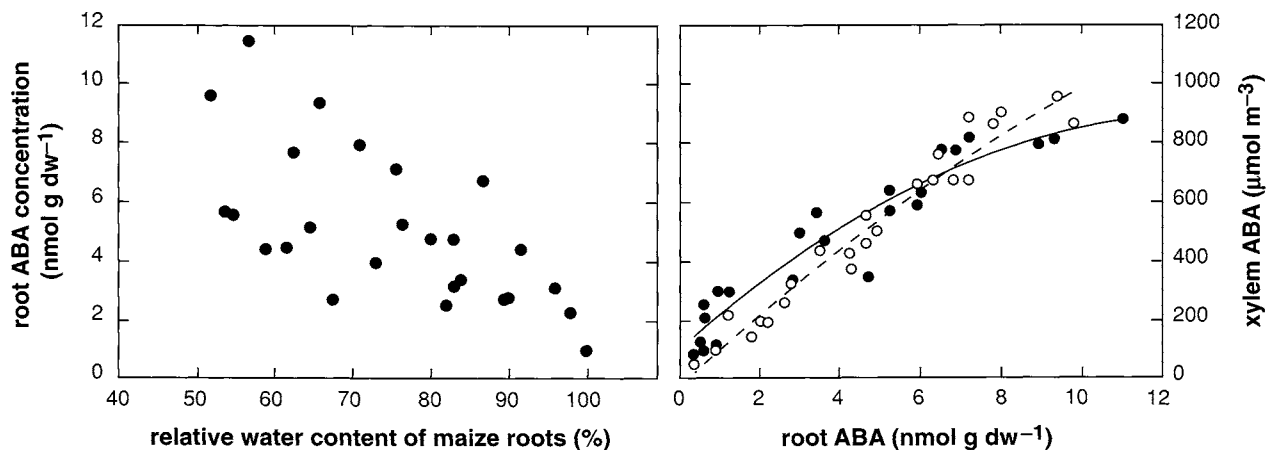


FIGURE 16-6 Accumulation of ABA in roots and in xylem sap as a result of soil drying. (Left) ABA content in roots of maize (*Zea mays*). Roots from 30-day-old seedlings were excised, air-dried to different relative water contents, and allowed to equilibrate in sealed containers for 3 h at room temperature. Each point represents one fraction ($\sim 1 \text{ g}$ fresh weight) of pooled root samples. Because the roots were excised before drying, the increase in ABA content most likely comes from enhanced synthesis. (Right) Relationship between root ABA content and xylem ABA concentration in *Z. mays* (open symbols) and *Leucaena leucocephala* (closed symbols) subjected to different degrees of soil drying. Xylem sap was collected using a pressure chamber. Each point represents an individual plant. The ABA content was measured using radioimmunoassay. From Liang *et al.* (1997).

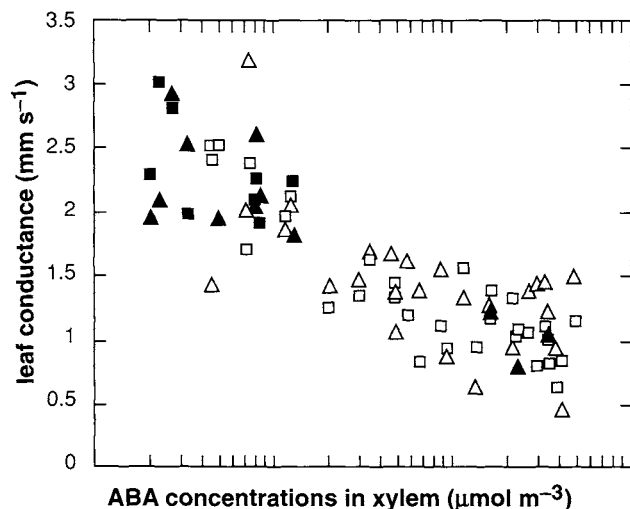


FIGURE 16-7 Effect of manipulating ABA concentration in xylem sap on stomatal conductance of leaves (abaxial epidermis) in maize. A series of ABA solutions were fed to a part of maize roots. Leaf conductance measurements were taken 24 (\blacktriangle , \triangle) or 48 (\blacksquare , \square) h after ABA feeding. Closed symbols are from plants fed with water. From Zhang and Davies (1990).

3.2. ABA and Stomatal Behavior

Irrespective of the source of ABA, whether it is root derived and transported through the xylem or synthesized in the leaf mesophyll, it accumulates in the apoplast surrounding the leaf epidermal cells. Guard cells sense this ABA and respond by stomatal closure. The architecture of guard cells and pH changes in the apoplast as a result of desiccation play important roles in stomatal pore behavior.

3.2.1. Guard Cells Are Accessible Only via Apostast

Guard cells, which are connected to neighboring epidermal cells along one wall only, hang free from subepidermal mesophyll cells and have no plasmodesmatal connections with either mesophyll or neighboring epidermal cells (Fig. 16-8). This freedom from contact with other cells is essential for the rapid volume changes that are the basis for stomatal pore opening or closing. However, it also means that in order to respond, the signal must reach the guard cells *via* the apoplast surrounding them.

3.2.2. Dehydration Causes a Rise in Apostast pH and a Redistribution of ABA in the Symplast vs Apostast

Wolfram Hartung and associates in Germany have suggested that drying of leaves or roots causes pH changes in the apoplast, resulting in a redistribution of ABA between the symplast and the apoplast. As

mentioned in Chapter 13, the pH of the cytosol is about 6.8–7.0, whereas that of the apoplast is usually much less, about 5.5–6.0. The low pH in the apoplast is maintained by the activity of proton ATPases (see Box 13-1 in Chapter 13). Dehydration leads to an inhibition of these ATPases. As a result, the pH of the apoplast in drying tissues rises to alkaline levels, creating a situation where intracellular ABA can diffuse into the apoplast. Experiments with excised barley leaves fed [^{14}C]ABA for 3 days showed the greatest accumulation of labeled ABA and its metabolite, phaseic acid, in the leaf apoplast (Fig. 16-9).

3.2.3. Apostast ABA Has to Be Metabolized in Order for Stomata to Reopen on Rehydration

On arrival in the leaf apoplast, ABA affects stomatal closure, but then, in order for stomata to reopen on rehydration, it is essential that excess ABA in the apoplast be metabolized quickly. Rehydration activates proton pumps, causing a drop in the apoplastic pH, which favors the uptake of ABA from apoplast into symplast where it is metabolized. Hartung and associates have also studied the capacity of epidermal and mesophyll cell protoplasts for ABA metabolism and found that, at pH 6.0, epidermal cells are far more efficient in metabolizing ABA to PA and ABA-glucosyl ester than mesophyll cells.

3.3. Leaf Growth, Photosynthesis, and ABA

Water deficit sooner or later leads to a cessation of leaf growth and photosynthesis, and several authors have linked these phenomena to a rise in xylem sap ABA. Leaf growth, compared to stomatal behavior, is a more complex phenomenon affected by numerous factors: ambient temperature, free water potential, leaf turgor, and the presence of wall-loosening enzymes, such as xyloglucan endotransglycosylases (XETs) and expansins. Whereas a high ABA content often acts as a growth inhibitor, no unique relationship between leaf growth inhibition and ABA concentration has been found. Indeed, many reports indicate that leaf growth can vary substantially against a background of a fairly constant ABA concentration. To complicate matters further, the developmental state of the leaf also plays a role. The sensitivity of young leaves to growth inhibition by ABA is much lower than that of older, still-growing leaves, and young vigorously growing leaves have often been shown to have a high ABA content. Thus, a high ABA concentration *per se* has little to do with growth.

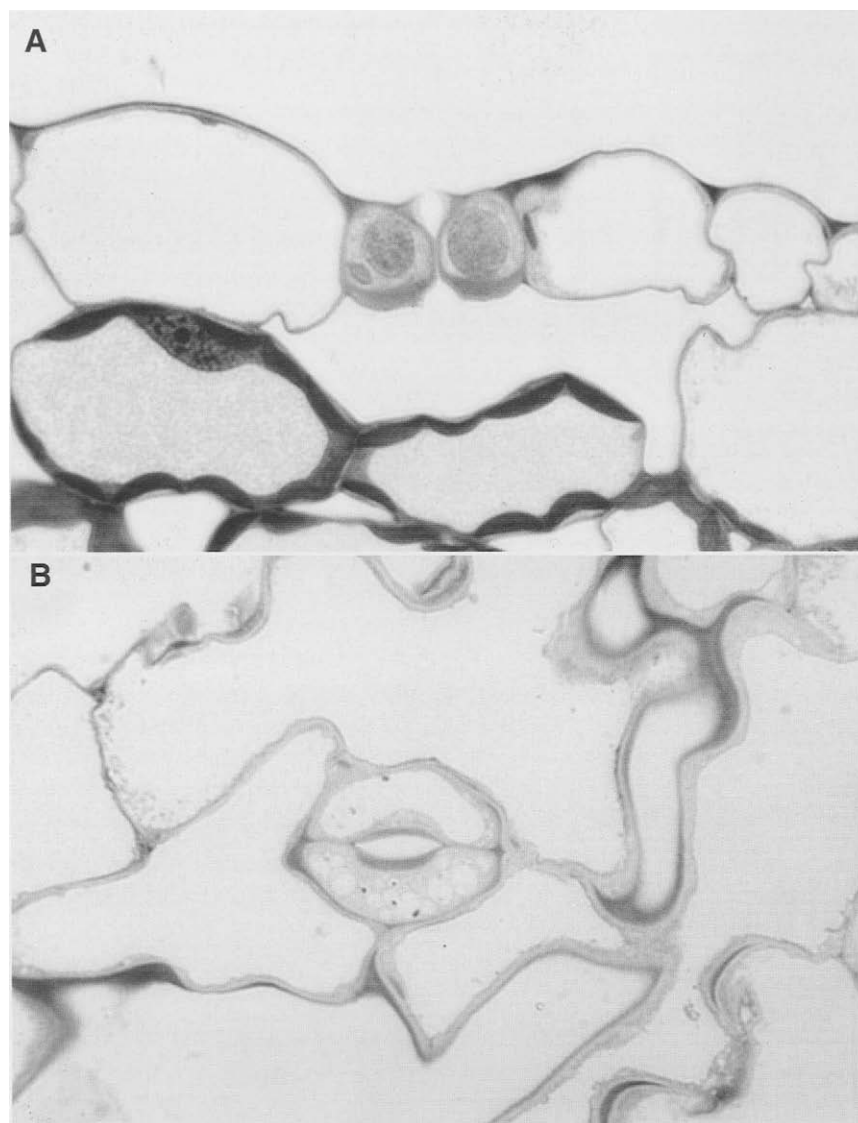


FIGURE 16-8 Vertical (A) and paradermal (B) sections of pea leaf through stomata. (A) Note that guard cells are connected to their neighboring epidermal cells along only one anticlinal wall and that they hang free above the mesophyll cells. Magnification, $\times 400$.

The relationship with photosynthesis is also uncertain. Water deficit leads to a decline in the steady-state level of the transcripts of the small subunit (*rbcS*) of ribulose biphosphate carboxylase/oxygenase (RUBISCO), the enzyme responsible for carbon fixation in photosynthesis. This decline is correlated with an increase in xylem sap ABA, but whether ABA is the direct cause of decline is not clear.

In summary, water deficit causes an increase in ABA synthesis in both root and shoot, and ABA concentrations in the xylem sap rise considerably above the levels in nonstressed plants. An increase in ABA concentration in the leaf apoplast is the major signal

for stomatal pore closure. This increase results from a change in apoplastic pH (itself a consequence of water deficit), which favors the movement of ABA from the symplast to the apoplast. On rehydration, ABA from the apoplast moves back to the symplast where it is metabolized to inactive products. The source of ABA, whether it is primarily root or shoot derived, or derived from both sources, is unclear. It is also uncertain whether ABA is the only signal or whether some other signal(s) (e.g., pH) may also participate. Water deficit also leads to an inhibition of leaf growth and photosynthesis, but the relationship with ABA is mostly correlative.

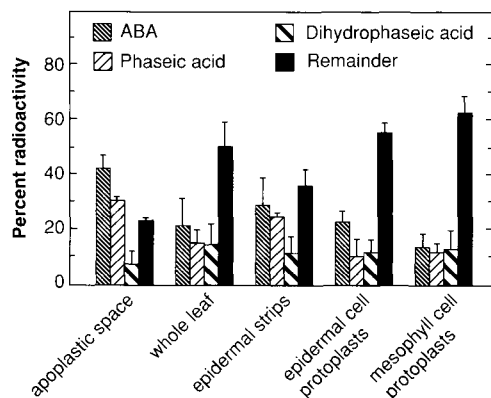


FIGURE 16-9 Relative amounts of labeled ABA and its metabolites in barley (*Hordeum vulgare*) leaves. Excised whole leaves were supplied [^{14}C] ABA for 3 days. Then labeled ABA and metabolites were measured in whole leaf, leaf apoplast, epidermal strips, and protoplasts obtained from epidermal and mesophyll cells. To obtain the apoplastic fraction, ABA-fed leaves were incubated in sorbitol (300 μM), a sugar alcohol that is taken up mainly in the apoplast and displaces ABA. Then they were put on a sieve and centrifuged under vacuum; the fraction collected in the vacuum tubes was used for analysis of ABA and metabolites. Data are given as a percentage of the total radioactivity in each sample. From Daeter and Hartung (1995).

4. FREEZING TOLERANCE

4.1. Chilling and Freezing Injuries and Cold Acclimation

Temperature is an important environmental factor in determining plant distribution on earth. Tropical or subtropical species and many cultivated crops, derived from tropical ancestors, such as maize, rice, tomato, cucumber, tobacco, and cotton, and many ornamentals, such as *Coleus*, are sensitive to cold temperatures. When these plants are exposed to 10–15°C, then show inhibition of growth and reduced photosynthesis and respiration. At lower temperatures (usually from 10–15 down to 0°C), leakage of solutes, discolorations and lesions on leaves, and even death may follow. This syndrome of changes, referred to as **chilling injury**, occurs at temperatures low enough to inhibit growth and cause injury, but not low enough to cause freezing. Some of these plants (e.g., maize and tobacco) are able to tolerate chilling temperatures if they are acclimated to cool temperatures over a period of a few days/weeks, but they do not survive freezing.

Plants growing in temperate climates suffer **freezing injuries** or are killed if exposed to freezing temperatures in summer months. However, these same plants are able to tolerate subzero temperatures in winter by being exposed to chilling temperatures (usually 2–10°C) during the fall months, a process known

as **cold acclimation** (also referred to as freeze tolerance or cold, frost, or winter hardening). For instance, winter rye (*Secale cereale*), one of the hardiest crop plants, is killed at –5°C if nonacclimated, but survives to –30°C if cold acclimated for a few weeks. In woody species that have been investigated, shortening of day length in late summer/early fall serves as the first trigger; it is followed by a low-temperature induction, which allows the trees to survive deep-freezing temperatures in the winter.

The ability to cold acclimate is under genetic and developmental control. Crosses involving compatible parents of differing cold resistance typically yield progenies that exhibit a continuous range of hardiness between the parental extremes, which suggests that winter hardiness is a quantitatively inherited trait involving multiple genes. In wheat, where most work has been done, at least 10 of the 21 pairs of chromosomes carry genes that influence winter hardiness. The ability to cold acclimate is also affected developmentally. The crown of the plant, consisting of the shoot apex and young leaves, is much more cold hardy and can tolerate much lower temperatures than the more mature older parts. Roots and underground stems are generally much less cold hardened than aerial parts because of the buffering action of the soil (Fig. 16-10).

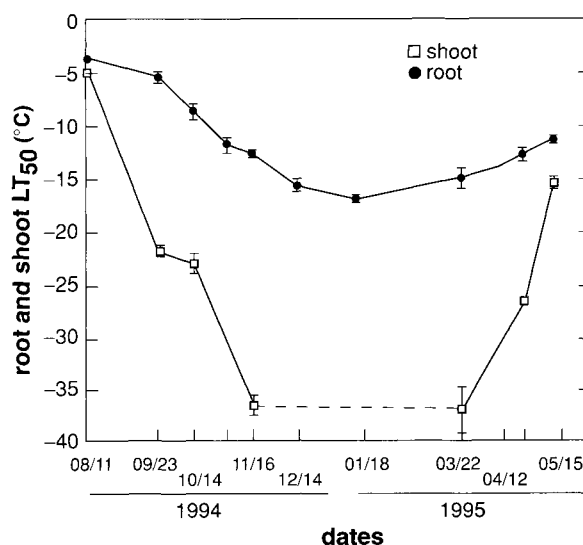


FIGURE 16-10 Cold hardiness of roots and shoots of sugar maple (*Acer saccharum*). Cold hardiness was measured by determining the LT₅₀ (for LT₅₀, see Box 2); lethality was measured by the discoloration of phloem and cambial regions. Cold hardiness was maximal from November to March and least between May and August. The shoot LT₅₀ was lower than –36°C between November 16, 1994, and March 22, 1995, but could not be evaluated precisely because the lowest temperature reached in the cold room was –40°C. From Bertrand *et al.* (1997).

4.2. Nature of Freezing Injuries

Both chilling and freezing injuries involve damage to cell membranes, but freezing injury involves more severe changes associated with ice formation. Ice formation itself is not particularly damaging, but the resultant dehydration is. Ice formation starts in extracellular space (apoplast) because the solute content of the apoplastic fluid is usually much less and the freezing point is correspondingly higher than that of the intracellular fluid. Ice formation results in a lower water potential in the extracellular space; consequently, water from inside the cell moves out into the apoplast, causing dehydrative damage to membranes and macromolecules. It has been estimated that, at -10°C , more than 90% of osmotically active water may transfer to the extracellular space, and the interior solute concentration may be as high as 5.0 osmolar.

Membrane damage consists of a change from liquid, semicrystalline to a gel phase and an associated loss of activity of intrinsic membrane proteins and enzymes (e.g., ATPases, carrier and channel proteins). These changes result in loss of selective-permeability properties of membranes and leakage of electrolytes, phenolics, and other stored solutes on thawing (see Box 16-2). In chill-sensitive species, this loss of osmotic responsiveness occurs at a higher temperature than in freeze-resistant species. Under severe winter conditions, intracellular ice is formed and is usually lethal. Large ice crystals cause mechanical damage to the membranes, a damage that is exacerbated by alternating freeze-thaw. Membranes may also adhere against the cell wall or against each other as a result of freezing.

Other injuries result from a buildup of toxic compounds because of a curtailment in aerobic respiration, production of reactive oxygen species (ROS), and per-

BOX 16-2 MEASUREMENT OF FREEZING INJURY

FREEZING INJURY IS USUALLY measured by exposing whole plants, plant parts (e.g., leaves), or protoplasts to a descending series of subzero temperatures (e.g., -2°C , -4°C , -8°C , etc.) in a thermostat or a cold room. The materials are held at any one temperature for a defined time and then are taken out and thawed. Tolerance to freezing is specified as the temperature at which 50% lethality (LT_{50}) is encountered. Lethality is measured by a lack of living processes, such as accumulation of vital dyes in vacuoles, cytoplasmic streaming, or growth, or by discoloration or browning of tissues, which results from leakage of phenolic compounds. Ions (electrolytes) stored in various compartments also leach out of the cells, and the extent of cellular damage can be estimated by conductivity measurements in what is known as the electrolyte leakage test (Fig. 16-11).

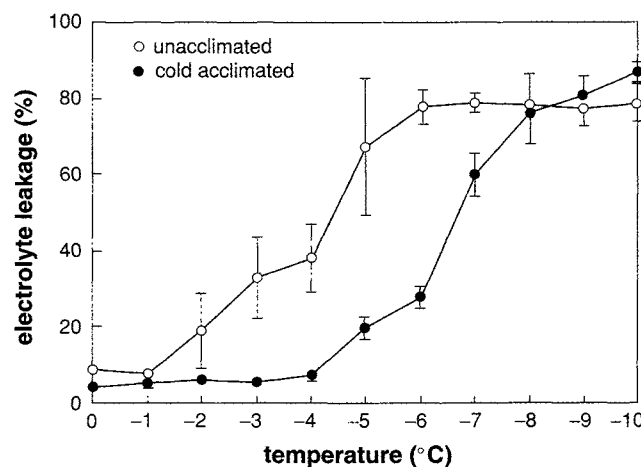


FIGURE 16-11 Leakage of electrolytes from mature *Arabidopsis* leaves before acclimation and after acclimation at 4°C for 7 days. Leaves were held at temperatures indicated for 30 min, thawed slowly on ice, and then placed in test tubes with deionized water and shaken overnight. After measurement of conductivity, the same leaves were placed in an autoclave at 121°C for 10 min. After cooling, conductivity was tested again. Percentage electrolyte leakage was calculated as the percentage of the conductivity before autoclaving over that after autoclaving. From Lee *et al.* (1999).

Many different types of lesions, whose severity varies with temperature, occur in membranes of unacclimated plants as a result of freezing. Some lesions occur even in membranes of acclimated plants exposed to freeze-thaw. They are not covered here and the interested reader is referred to specific papers cited in the reference section.

oxidation of polyunsaturated fatty acids in membrane lipids, a potential threat because the unsaturation of membrane fatty acids is enhanced by cold acclimation (see Section 4.3.1). Denaturation of proteins can also occur as part of dehydrative damage.

4.3. Role of Cold Acclimation in Freezing Tolerance

Since a sudden shock of a freezing temperature to unacclimated plants kills them, some changes must occur during cold acclimation. These changes occur at multiple levels. The best documented changes include (i) alterations in lipid composition of the membranes; (ii) accumulation of osmolytes, especially low molecular weight organic compounds; (iii) synthesis of anti-freeze proteins; and (iv) synthesis of proteins, which are thought to protect membranes and macromolecules against dehydrative damage. In addition, many trees develop the capacity to supercool. Also, several proteins with possible functions as chaperons, or antioxidants, or related to pathogenesis (PR proteins) are synthesized. Some of these strategies, i.e., the synthesis of osmolytes and various proteins involved in controlling dehydration damage, antioxidants, and PR proteins, are shared by plant responses to drought and salinity; hence, they are considered separately in Sections 5 and 6. Others that are unique to freezing tolerance, such as an increase in membrane-lipid unsaturations, synthesis of antifreeze proteins, and supercooling, are discussed in this section.

4.3.1. Changes in Membrane Lipids

Changes in membrane lipids are supported by several lines of evidence. In a survey of 74 plant species, it was found that those that are chilling tolerant have a greater proportion of unsaturated fatty acids in their membrane lipids than those that are chill sensitive. The explanation is that unsaturations provide a greater degree of fluidity to lipids; consequently, cell membranes remain functional at lower temperatures. As explained elsewhere, desaturases are enzymes that introduce double bonds at specific locations in fatty acid chains (see Box 12-2, Chapter 12). Activities of desaturase enzymes increase during acclimation. The relative proportions of polyunsaturated to saturated

and *trans*-monounsaturated fatty acids (the so-called "high-melting point" fatty acids), as well as the relative amounts of linoleic (18:2) or linolenic (18:3) acid in membrane lipids, increase as plants acclimate to cold temperatures. Many cDNAs and genes encoding desaturase enzymes have been cloned. The expression of a desaturase gene, *FAD8*, in *Arabidopsis*, is specifically induced under cold acclimation. In addition, the relative ratios of phospholipids to sterols in membranes increase with cold acclimation, a change that is associated with enhanced fluidity.

Fatty acid desaturase (*fad*) mutants, which are defective in a particular desaturase, confirm the role of unsaturated fatty acids in freezing tolerance. For example, the *fad2* mutant of *Arabidopsis* is defective in 18:1 desaturase, which converts oleic acid to linoleic acid in the eukaryotic pathway of lipid synthesis. While the *fad2* mutant has a normal phenotype when grown at 22°C, it is stunted in elongation growth at 12°C and is killed at 6°C. The wild-type plants, although inhibited in growth, survive the lower temperatures.

Tobacco plants are intermediate in chilling sensitivity between squash and *Arabidopsis*. Glycerol-3-phosphate acyltransferases (GPATs) are enzymes that transfer a fatty acid chain to glycerol-3-phosphate in the plastids or cytosol. The specific isoform of GPAT determines whether saturated fatty acids or unsaturated fatty acids will be added to the C-2 of glycerol; in the chill-sensitive squash, the isoform adds saturated fatty acids, whereas in the chill-resistant *Arabidopsis*, it adds unsaturated fatty acids. Thus, tobacco plants, which show intermediate chill sensitivity, transformed with the gene for GPAT from squash show a lower concentration of *cis*-unsaturated molecular species in phosphatidylglycerol and an enhanced sensitivity to chilling than untransformed controls. In contrast, those transformed with the GPAT cDNA from *Arabidopsis* show a higher concentration of *cis*-unsaturated species and a greater chill resistance (Fig. 16-12). These experiments clearly show that the fatty acid composition of a species has a role in chilling sensitivity and that species that are chill tolerant have a higher ratio of unsaturated/saturated fatty acids than species sensitive to chilling because of the specific isoforms of enzymes that they carry.

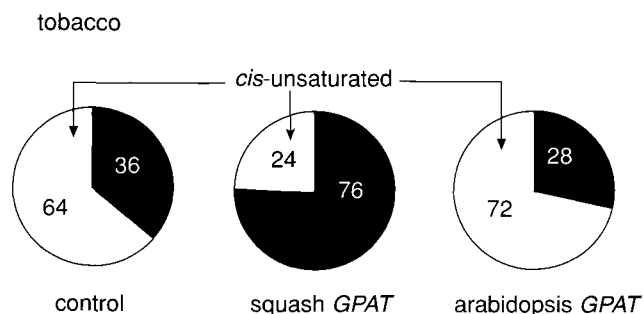


FIGURE 16-12 Change in relative levels of *cis*-unsaturated molecular species of phosphatidylglycerol (PG). Tobacco plants were transformed to overexpress *GPAT* cDNAs from either squash or *Arabidopsis*. Clear areas represent the percentage of PG with unsaturated fatty acids in the transformants. The shaded areas represent the sum of saturated and *trans*-monounsaturated fatty acids, which are thought to be "high melting point" fatty acids. From Nishida and Murata (1996).

4.3.2. Antifreeze Proteins (AFPs)

Antifreeze proteins occur in a wide range of organisms that are exposed to freezing temperatures in their native habitat: fish, insects, fungi, and plants. They represent a variety of proteins that seem to have evolved independently in different taxa.

Many plants, both dicots and monocots, produce AFPs. Those in cereal crops, such as winter varieties of rye, wheat, and barley, have been better investigated than in other plants. In winter cereals, AFPs are synthesized during cold acclimation in the field or under laboratory conditions. After synthesis, they are targeted to the extracellular space and are deposited selectively

in the apoplast, as shown by immunostaining with antibodies. Because they are present in the apoplast, they are extracted easily. Leaves can be soaked in buffer (20 mM ascorbic acid), placed in cones, and centrifuged; the crude extract can then be purified.

The capacity of plant AFPs to depress the freezing temperature (thermal hysteresis) is low (only 1–2°). A 67-kDa AFP purified from cold-acclimated bittersweet nightshade (*Solanum dulcamara*) produced only about 0.3°C of thermal hysteresis at a concentration of 10–30 mg ml⁻¹. In contrast, insects that avoid freezing produce AFPs that, at similar concentrations, depress the freezing point by up to 7°C. The main action of plant AFPs lies in preventing the growth of ice crystals by interacting directly with ice. They adsorb onto the surface of ice crystals and prevent further accretion of water molecules along the bound surface (Fig. 16-13), a form of protection that is potentially operative at a wider temperature range than depression of the freezing point.

In winter rye, purified AFPs belong to certain classes of glucanases, chitinases, and thaumatin-like proteins, all enzymes that have antipest or antifungal properties. The same proteins are also synthesized in summer months or in nonacclimated plants and are deposited in the apoplast, but lack the antifreezing properties. Hence, it is thought that AFPs in cereals represent isozymes of pathogenesis-related proteins that are produced on cold acclimation.

The dicots examined so far that are cold acclimated and freezing tolerant, such as kale, spinach, and oilseed rape, do not accumulate apoplastic AFPs, or do so to very low levels, when exposed to chilling temperatures.

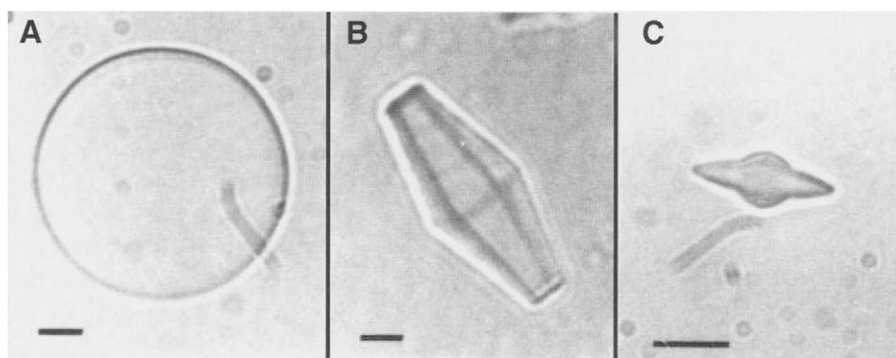


FIGURE 16-13 Prevention of ice crystal growth by antifreeze proteins. (A) Crystal growth in water molecules adds preferentially onto the circumference to form a larger, circular, flat crystal. (B) Water plus a purified AFP, a glucanase, from winter rye; the crystal growth around the circumference is prevented, and the crystal grows by the addition of water molecules along the basal plane. The crystal is hexagonal in cross section, and pyramidal faces are flat, indicating binding along one plane of the ice crystal lattice. (C) Water plus a crude, unpurified extract of apoplastic proteins from cold-acclimated winter rye leaves. The extract contains multiple AFPs, each of which may bind to different planes of the ice crystal lattice to form complex facets; crystals are hexagonal in cross section. In all photos the basal plane is perpendicular to the page. From Griffith *et al.* (1997).

The mode of action of AFPs in dicots is not known, but if they are accumulated intracellularly, they may be involved in supercooling and preventing nucleation of ice (see below).

4.3.3. Supercooling or Restricted Ice Formation

Woody species use two strategies to survive subfreezing temperatures: supercooling or restricted ice formation. Supercooling allows an aqueous solution to reach temperatures below the theoretical freezing point without ice formation. Ice formation requires aggregation of water molecules at what are known as nucleation centers to begin ice crystal formation. Once formed, ice crystals grow by the accretion of new water molecules from the neighboring environment. Lack of nucleation centers results in supercooling. Studies on trees growing in eastern Canada, the north eastern United States, and in the Rockies in Colorado indicate that these plants tolerate subfreezing temperatures in winter by supercooling. Because supercooled water is still liquid, dehydrative damage is minimal. For pure water, the theoretical limit to supercooling is about -38.5°C —at that temperature, ice crystals form spontaneously with a big release of latent heat of fusion, which can be measured. Species that use supercooling can tolerate temperatures to $\sim -40^{\circ}\text{C}$, which limits their geographic distribution. How trees develop the capacity for supercooling is unknown, but the capacity to supercool must develop during the fall months.

Trees and shrubs in northern Canada and Alaska, which are exposed to much lower temperatures than -40°C , survive by allowing ice formation to occur in the apoplastic space of outer bark tissues. Such ice formation is believed to act as a thermal barrier, and plants survive dehydrative damage by restricting the loss of cellular water from inner tissues.

4.4. ABA Is Involved in Cold Hardening

Evidence that ABA is involved in the cold acclimation of plants that are genetically competent to do so comes in very much the same way as the evidence for ABA involvement in tolerance to water stress.

In many plants, cold acclimation has been correlated with a transient increase in the endogenous ABA concentration. For example, in *Solanum commersonii*, a plant that cold acclimates, ABA levels increase transiently in response to low temperature, but they do not in *S. tuberosum* (potato), which does not cold acclimate. Similar results have been reported from several other plants, including wheat (see Fig. 16-2), spinach, *Arabidopsis*, alfalfa, some cacti, and trees. In a study on sugar maple trees, it was shown that the ABA concentration in xylem sap increased during cold acclimation and reached

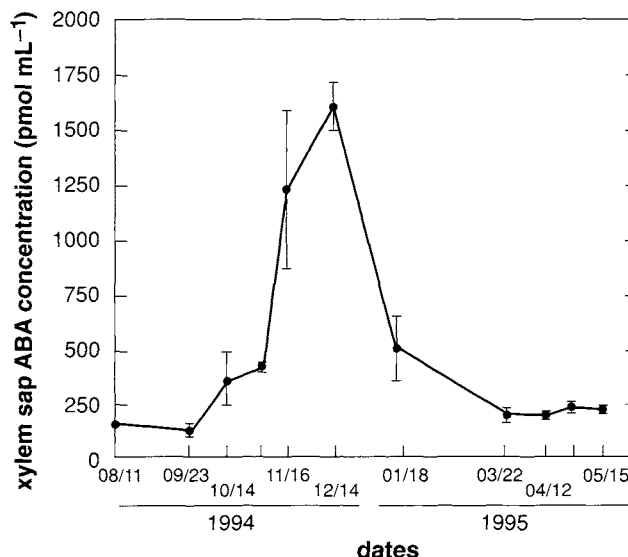


FIGURE 16-14 Concentration of ABA in the xylem sap of sugar maple (*Acer saccharum*) during cold hardening and dehardening. One-year-old trees of sugar maple were cold hardened in the field. Xylem sap was obtained from cut stumps and partially purified before determination of ABA content by radioimmunoassay. Mean \pm SE for $n = 3$. From Bertrand *et al.* (1997).

maximal levels (ca. $1600 \text{ pmol} \cdot \text{mL}^{-1}$) just before full acclimation (Fig. 16-14).

An exogenous application of ABA at warm temperatures ($20\text{--}25^{\circ}\text{C}$) to whole plants that are genetically competent to cold acclimate increases their freezing tolerance approximately to the same level as exposure to low temperature (Fig. 16-15). Similar data are known for suspension cell cultures (e.g., from tissue of *Bromus inermis*, *Brassica napus*). Moreover, ABA synthesis mutants, such as *aba1* in *Arabidopsis*, fail to cold acclimate, but do so if supplied ABA (Fig. 16-15B). Figure 16-15C also illustrates the phenomenon of cross protection. *Arabidopsis* plants of the wild type which are exposed to drying conditions show an increase in their freezing tolerance.

More support for a role of ABA in cold acclimation comes from the induction of genes involved in the synthesis of certain osmolytes, as well as many proteins associated with dehydration tolerance. These topics are covered elsewhere in this chapter.

4.5. Exposure to Cold Temperature Acts as a Cue to Many Other Developmental Processes

Exposure to chilling temperatures slows growth. It also acts as a “switch” for many other developmental processes, including bolting in biennials; induction of winter dormancy in buds of trees and shrubs in

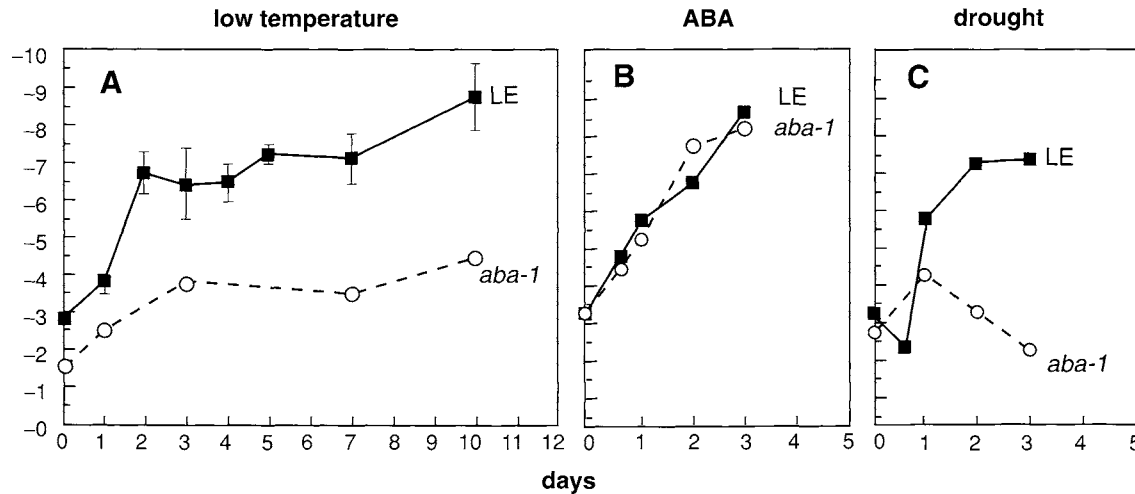


FIGURE 16-15 Induction of freezing tolerance in ecotype Landsberg erecta (LE, wild type) and an ABA synthesis mutant *aba1* of *Arabidopsis thaliana*. Plants were grown under controlled conditions, held either at (A) low temperature (4/2°C, day/night) or at (B) 22°C and supplied ABA (60 μM) or (C) desiccated to 70% relative humidity for the specified number of days, and the LT₅₀ measured. The data show that the wild-type plants are much more cold tolerant than the *aba1* mutant; though the latter on being supplied ABA becomes just as tolerant as the wild-type. Panel C shows that the wild-type plants show a higher cold tolerance if dehydrated for 2 days, but the *aba1* mutant does not. From Mäntylä *et al.* (1995).

temperate climates; and development of underground storage organs, such as bulbs and tubers. Chilling, or cold stratification, is required to break seed dormancy in many plants growing in temperate climates. In some of these phenomena, such as bud dormancy, photoperiod is a primary regulator. Another important phenomenon regulated by cold temperature is vernalization: induction of flowering in plants after exposure to a period of chilling temperatures. The phenomenon is common in winter varieties of cereals and other plants. Accordingly, many genes are expressed by exposure to low temperature, only some of which are related directly or indirectly to cold acclimation.

In summary, cold acclimation is the key to freezing tolerance, but only plants that are genetically competent to do so cold acclimate. The most notable damage as a result of freezing occurs in cell membranes. In unacclimated plants, the membranes undergo a change from a liquid to a gel phase with a concomitant loss of function of many intrinsic membrane proteins. As a result, membranes lose their selective permeability and solutes leak out on thawing. Other damages may result from denaturation of proteins and from buildup of toxic substances and free oxygen radicals. Cold acclimation prepares the cells/plants for freezing temperatures by changes in membrane lipid composition, which include an increase in the phospholipid/sterol ratio and an increase in the amounts of polyunsaturated fatty acids (18:2 or 18:3) relative to saturated and *trans*-monounsaturated fatty acids. Other changes, which appear to be more species specific, include the synthesis of antifreeze

proteins and the acquisition of a capacity to supercool. ABA is involved in the development of freezing tolerance. ABA concentrations increase transiently in plants exposed to low temperatures, application of ABA to plants and tissues at warm temperatures improves their freezing tolerance, and ABA synthesis and ABA-insensitive mutants are impaired in their capacity to cold acclimate. Other changes during cold acclimation pertain to the synthesis of osmolytes and many different kinds of proteins, topics which are covered in Sections 5 and 6 below.

5. ACCUMULATION OF OSMOLYTES AND STRESS TOLERANCE

Drought, salinity, and freezing all lead to cellular dehydration and attendant damage to cell membranes and macromolecules. One of the major ways that plant cells protect themselves against loss of water is by an accumulation of ions or molecules, which increase the osmotic pressure and, thus, lower the free water potential (Ψ) of the cells.¹ Compounds that restrict water

¹The concentration of the osmolytes in a cell, or its osmotic pressure (OP) is inversely related to its free water potential (Ψ); the greater the OP, the lesser or more negative the value of Ψ . The water potential of pure water is 0 mPa; hence, the presence of any solute in water reduces Ψ to a negative value, e.g., -0.2 mPa. The movement of water occurs in response to its concentration gradient, i.e., from a region of less negative (higher) to a region of more negative (lower) Ψ .

loss and attendant loss in cell turgor are referred to as osmolytes and the phenomenon as osmotic compensation or osmotic adjustment (Fig. 16-16). Osmotic compensation allows cell growth, photosynthesis, and other metabolic phenomena to continue while the stress conditions last.

Osmolytes include ions and low molecular weight organic compounds. Ions are accumulated only in some plants, mostly halophytes (e.g., ice plant). They are selectively accumulated in vacuoles because a high ionic concentration in the cytosol inhibits the activities of many cytosolic enzymes (see Fig. 16-18). Both the rate of enzyme activity and the K_m for the substrate are thought to be affected. Specific Na^+/H^+ antiporters coupled to ATPases or pyrophosphatases transport Na^+ ions to vacuoles; other ions (e.g., Cl^-) are transported via specific channel proteins (for carrier and channel proteins, see Box 13-1 in Chapter 13). Accumulation of Na^+ occurs naturally in halophytes, but transgenic glycophytes can also accumulate Na^+ in their vacuoles. In *Arabidopsis*, a cDNA encoding a Na^+/H^+ antiport protein was overexpressed, and the transgenic plants were able to tolerate much higher levels of NaCl in water than the wild-type plants (Fig. 16-17).

Low molecular weight organic compounds include sugars, sugar alcohols, and nitrogen- or sulfur-containing compounds. These solutes are accumulated in the cytosol and, in some cases, in organelles. Their accumulation, even at relatively high concentrations, does not inhibit the activities of enzymes (Fig. 16-18).

Hence, these osmolytes are referred to as compatible solutes or compatible osmolytes. They are thought to offer protection against desiccation in two ways. First, their accumulation decreases the cytosolic-free water potential, which restricts the loss of water to the cell exterior (in halophytes accumulating salt in vacuoles, it also restricts the loss of water to the vacuole). Second, they protect protein complexes in organelles and cytosol against dehydration damage, presumably by keeping them hydrated. Hence, they are also called osmo- or cryoprotectants. Other functions have been postulated (e.g., some of the charged osmolytes may buffer cellular redox potential). The accumulation of compatible osmolytes is a common feature in widely divergent groups of organisms, including bacteria, algae, vascular plants, invertebrates, and vertebrates, which indicates that their accumulation is an evolutionarily conserved trait. However, no single osmolyte is accumulated in all groups or in any one group. Indeed, different osmolytes may be accumulated in different members of the same plant family.

Sucrose is the most commonly accumulated sugar in plants, although hexoses (glucose and fructose), oligosaccharides (e.g., verbascose, stachyose, raffinose), and sugar alcohols (e.g., mannitol, sorbitol) are also accumulated. Sucrose accumulation occurs in many plants after cold acclimation or drought. In winter wheat, activities of the major enzymes in sucrose metabolism, sucrose phosphate synthase and acid invertase, increase several fold on exposure to low temperatures. In *Arabidopsis*, the transcripts of one isoform of sucrose

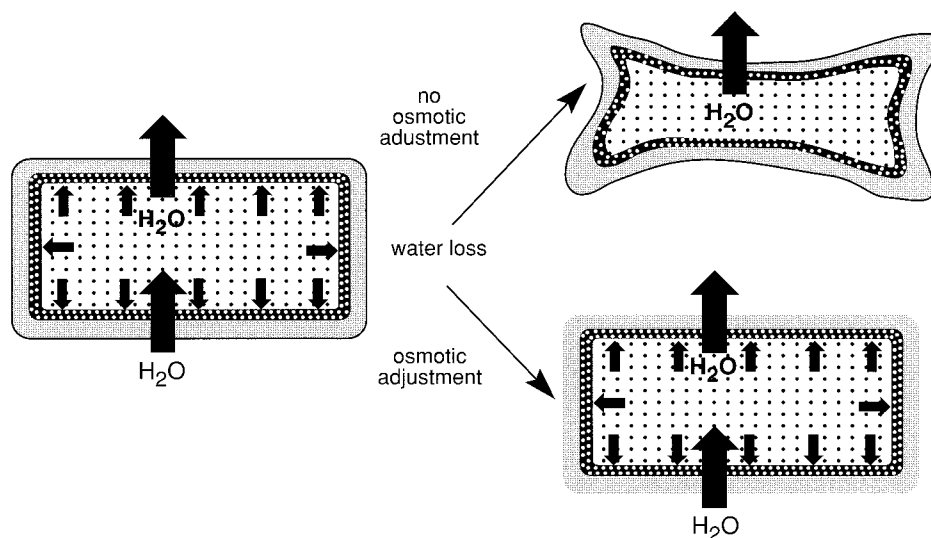


FIGURE 16-16 Schematic drawings showing osmotic compensation. When water is plentiful, water enters and leaves the cell, but the cell maintains its turgor pressure (small black arrows against the wall). When water potential in the ambient environment is more negative than in the cell, water from the cell moves out, causing water stress. An increase in intracellular osmolytes decreases the water potential in the cell and prevents water from moving out.

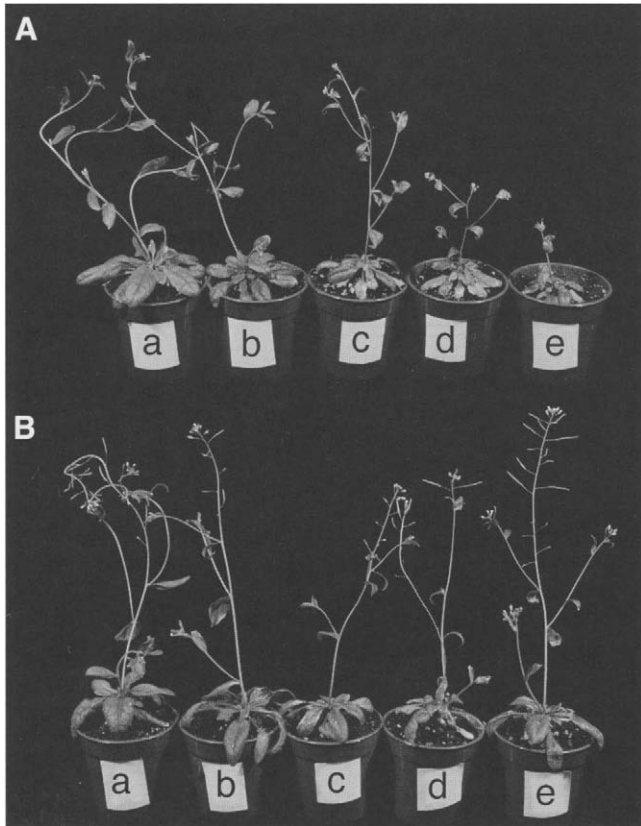


FIGURE 16-17 Wild-type and transgenic *Arabidopsis* plants exposed to salinity stress. Transgenic plants were transformed to over-express the gene for a Na^+/H^+ antiport (*AtNHX1*). Wild-type and transgenic plants were divided into five groups, a through e, and watered with a nutrient solution supplemented with NaCl. The concentrations of NaCl supplement were increased stepwise by 50 mM every 4 days for each group to the indicated maximum: (a) control, (b) 50 mM NaCl, (c) 100 mM NaCl, (d) 150 mM NaCl, and (e) 200 mM NaCl. Wild-type plants (A) and the transgenic line (B). With permission from Apse *et al.* (1999), ©1999 American Association for the Advancement of Science.

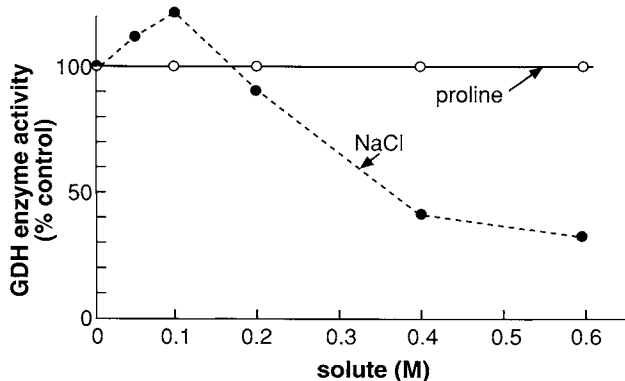


FIGURE 16-18 Effects of increasing concentrations of NaCl or proline (a compatible osmolyte) on the activity of glutamate dehydrogenase (GDH) from the halophyte *Triglochin maritima*. Enzyme activity is expressed as a percentage of control (without added salt or proline). From Stewart and Lee (1974).

synthase increased on exposure to cold and drought and were accompanied by an accumulation of soluble sugars. In the ice plant, which accumulates sodium chloride in the vacuoles, a sugar alcohol, D-pinitol, is accumulated in the cytoplasm. Trehalose is one of the most efficient sugars as an osmoprotectant and is common in many organisms, but is rare in plants.

Nitrogen- or sulfur-containing compounds include amino acids (e.g., proline, glycine, taurine), quaternary ammonium compounds (e.g., glycine betaine or simply betaine), tertiary sulfonium compounds (e.g., dimethylsulfoniopropionate), and polyamines (e.g., spermidine). Proline and betaine are the most commonly accumulated osmolytes in halophytes and in some crop plants such as barley and spinach. Proline is derived from glutamate in a two-step reaction catalyzed by Δ^1 -pyrroline-5-carboxylate synthetase (P5CS) and pyrroline-5-carboxylate reductase (P5CR). (An alternate pathway from ornithine via P5CR is also known.) On rehydration or as part of feedback regulation, it is converted in a reverse set of reactions to glutamate by proline dehydrogenase (ProDH) and P5C dehydrogenase (P5CDH) (Fig. 16-19). Betaine is synthesized by a two-step oxidation of choline via the intermediate betaine aldehyde. In plants, the two enzymes catalyzing the steps are choline monooxygenase (CMO) and betaine aldehyde dehydrogenase (BADH). They are localized in the soluble fraction in plastids (e.g., spinach and sugar beet), peroxisomes (barley), or cytoplasm. Bacteria use different enzymes to produce betaine aldehyde, choline dehydrogenase (CDH in *Escherichia coli*), or choline oxidase (COD in the soil bacterium, *Arthobacter globiformis*); betaine aldehyde is subsequently converted to betaine by BADH.

The role of proline and betaine as compatible osmolytes in protection against dehydrative damage due to salinity, drought, or freezing is well established. To give a few examples, transgenic tobacco plants expressing P5CS from mothbean (*Vigna aconitifolia*) synthesize and accumulate as much as 10-fold more proline than control plants and show greater tolerance to dehydration, salt treatment, or freezing. Suppression of proline degradation in *Arabidopsis* by introducing the proline dehydrogenase (*ProDH*) cDNA in an antisense orientation enhances the tolerance of transgenic plants to freezing and high salinity. Salt-tolerant lines of tobacco (*Nicotiana tabacum*) have been generated from cells surviving in suspension cultures kept on a medium with 170 mM NaCl. These lines, under saline conditions, accumulate proline, polyamines (putrescine and spermidine), and betaine and survive much higher temperatures and salinity (up to 340 mM NaCl) than wild-type cells, which do not accumulate these osmolytes. Tobacco and

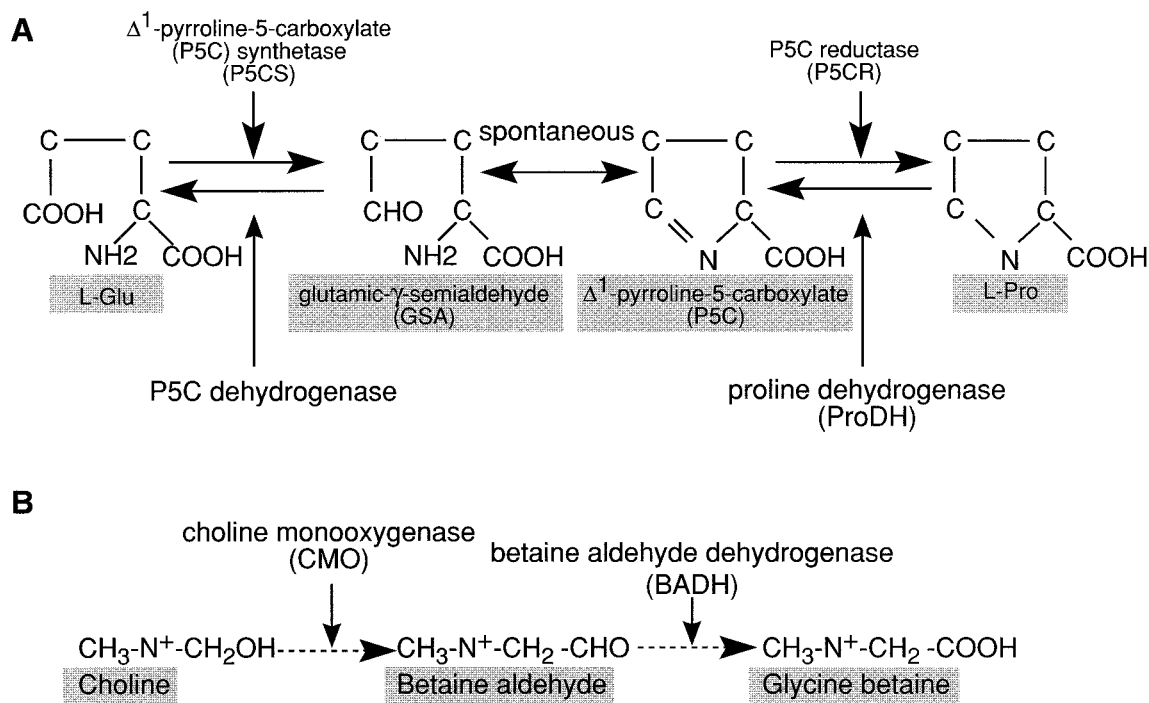


FIGURE 16-19 Synthesis of proline and betaine in plants [A from Yoshida *et al.* (1997) and B from Rhodes and Hanson (1993)].

rice plants normally do not accumulate betaine, but do so on transformation with a bacterial gene (*CDH*) or barley *BADH*, respectively, and show significant tolerance to salt, cold, and heat stress.

The synthesis of proline is induced not only by drought, salinity, or low temperature, but also by ABA. In *Arabidopsis*, the accumulation of *P5CS* transcripts in salt-treated seedlings is mediated via ABA, and a mutant deficient in ABA (*aba2-2*) does not accumulate proline under water stress, but does so if supplied ABA. For other osmolytes, the situation is not clear, but some are thought to be induced independently of ABA (e.g., gene encoding *BADH* enzyme in betaine synthesis, *AtNHX1* gene in *Arabidopsis* encoding a Na^+/H^+ antiporter).

6. STRESS-INDUCED GENE EXPRESSION IS COMPLEX AND VARIED

While expression of many genes associated with growth and photosynthesis is downregulated in plants under stress, at least temporarily, the expression of a variety of other genes is upregulated. The common damaging effects of dehydration under drought, high salinity, or freezing include increased salt concentra-

tion, inactivation/denaturation of proteins, coagulation of macromolecules and membranes that are normally kept separated by water, and an increase in concentrations of toxic compounds, such as ethanol and reactive oxygen species (ROS). Cold acclimation also involves synthesis/induction of enzymes involved in lipid metabolism and, at least in some plants, synthesis of antifreeze proteins. Several different kinds of proteins, including enzymes for altered metabolic pathways, are required to perform these functions, and many different types of genes are expressed in response to any one stress. Many of the genes expressed are common to two or more types of stresses, whereas others are unique to one stress. In addition, many of the stress-related genes are induced by exogenous ABA in unstressed plants. An analysis of these genes as to their inducing factor, whether the gene expression is mediated via endogenous ABA, and to work out the signaling pathways are major enterprises that have engaged the attention of many researchers since the mid-1980s and, although we do not have answers to many questions, the framework of how plants respond to stress is gradually being put in place.

In the following, some general features of stress-induced gene expression are highlighted, including the roles of some of the encoded proteins.

TABLE 16-1 A Representative List of Proteins of Known Function That Are Synthesized/Accumulated during Cold Acclimation

Functions	Examples
Stress tolerance	Fatty acid desaturase (FAD8) Δ^1 -Pyrroline-5-carboxylate synthetase (P5CS)
Transport of water/ osmolytes	Membrane-integral proteins, such as aquaporins
Signaling	Protein kinases, MAP kinases, 14-3-3 proteins
Involved in secondary effects	Heat shock proteins (HSPs)—chaperons, repair/salvage of denatured proteins; superoxide dismutases (SODs)—protection against reactive oxygen species; alcohol dehydrogenase (ADH)—an enzyme used in anaerobic metabolism phenylalanine ammonia lyase (PAL), chalcone synthase (CS), chitinases, glucanases—plant defense

TABLE 16-2 Representative Groups of Hydrophilic Polypeptides Encoded by Cold-Responsive Genes Induced during Cold Acclimation^a

Group	Plant	Polypeptide(s)
COR6.6	<i>Arabidopsis</i>	COR6.6/KIN1, KIN2
	<i>Brassica napus</i>	Kin1
	<i>Brassica rapa</i>	Kin
COR15a	<i>Arabidopsis</i>	COR15a, COR15b
	<i>B. napus</i>	BN115, BN26, BN19
	<i>Brassica oleracea</i>	BoCOR
COR78	<i>Arabidopsis</i>	COR78/LTI78/RD29a, LTI65/RD29b
	<i>Spinacea oleracea</i>	CAP160
WCS19	<i>Triticum aestivum</i>	WCS19
	<i>Hordeum vulgare</i>	CR1
CORa	<i>Medicago sativa</i>	CORa
CAS15a	<i>M. sativa</i>	CAS15a, CAS15b
COR47 (LEA II)	<i>Arabidopsis</i>	COR47/RD17, LTI29/ERD10, LTI30, ERD14, RAB18
	<i>H. vulgare</i>	DHN5
	<i>M. falcata</i>	CAS18, CAS17
	<i>Poncirus trifoliata</i>	COR19, COR11
	<i>S. oleracea</i>	CAP85
	<i>T. aestivum</i>	WCS120, COR39, WCS66, WCOR726, WCOR80, COR410
	<i>Vaccinium corymbosum</i>	BBDHN1
	<i>H. vulgare</i>	HVA1
HVA1 (LEA III)	<i>T. aestivum</i>	WCOR615

^aThe nomenclature of genes induced under stress is highly varied, perhaps because of the large number of researchers and the diversity of inducing stimuli. Many genes or cDNAs are named after the inducing agent, e.g., dehydration (DHN), responsive to desiccation (RD), early dehydration-inducible (ERD), low temperature induced (LTI), low temperature responsive (LTR), cold regulated (COR), cold induced (KIN), or responsive to abscisic acid (RAB). In some instances, they are named after the developmental stage at which they were first discovered, e.g., late embryogenesis abundant (LEA) genes. In some cases, they are named after the labeling pattern of their encoded product, e.g., early methionine-labeled (Em) gene. Proteins encoded by these genes/cDNAs are written in uppercase letters but are not italicized. Modified from Thomashow (1998).

6.1 Many Genes Are Expressed by Any One Factor

Many genes are induced during cold acclimation. Table 16-1 shows a representative sampling of proteins of known function, which can be divided into at least two groups. Proteins in one group directly participate in freezing tolerance (e.g., FAD8, P5CS). Mutants defective in these proteins are impaired in their capacity to tolerate freezing. Proteins in the other group (e.g., HSPs, ADH, PAL, chitinases) are involved in protection against secondary effects of stress. Mutations in genes encoding them do not affect the capacity for stress tolerance directly. Still another group of proteins (e.g., protein kinases, MAP kinases, 14-3-3 proteins) have a known function in signaling (see Chapter 25), but whose role in stress-related signaling has not been dem-

onstrated. Table 16-2 lists the proteins thought to function in protecting membranes and macromolecules against dehydrative damage, as AFPs, or other proteins that may enhance freezing tolerance. Many of these proteins fall into the LEA group of proteins, whereas others are novel proteins.

Tables 16-1 and 16-2 pertain to low-temperature-induced proteins, but similar lists with some common, some additional, proteins can be compiled for responses to osmotic stress caused by salinity or drought. The known or deduced functions of some major classes of proteins whose genes or cDNAs have been isolated from plants exposed to water deficit or salinity or low temperature are outlined in Box 16-3, irrespective of whether they are also induced by ABA, although many undoubtedly are. Box 16-3 is useful because many of these proteins are referred to in Chapters 18 and 20.

BOX 16-3 FUNCTIONS OF STRESS-RELATED PROTEINS

MOST OF THE PROTEINS described here are known from sequence data of their cDNAs. Their functions are either deduced from their postulated structures or inferred from comparisons with other proteins in data bases.

1. LEA and other hydrophilic proteins. Late embryogenesis abundant (LEA) proteins were first discovered in 1986 in a study of embryogenesis in cotton seeds, but their induction is not restricted to embryo tissues. Many LEA proteins are synthesized in vegetative tissues, leaves, stems, and roots in response to water deficit due to drought, high salt concentration, freezing, flooding, and O₂ deficiency (anoxia or hypoxia); many are induced in nonstressed tissues supplied exogenous ABA.

LEA proteins belong to several immunologically distinct groups. Three groups, I, II, and III, characterized by prototype cotton LEA proteins, are listed in Table 16-3. They have some striking features: (i) They occur predominantly, but not exclusively, in the cytosol and, while their concentrations vary, they can accumulate in substantial amounts in desiccating tissues. (ii) They have a strongly biased amino acid

TABLE 16-3 Hydrophilic Polypeptides Encoded by Genes Expressed under Drought, Salt Stress, or Cold Acclimation^a

Hydrophilic polypeptides	Example	Notable structural motifs
LEA I (cotton D19)	Em (wheat)	Random coil
LEA II (dehydrins) (cotton D11)	WCS120 (wheat)	Amphipathic α helix
LEA III (cotton D7)	HVA1 (barley)	Amphipathic α helix
Novel polypeptides	COR15am (<i>Arabidopsis</i>) CAS15 (alfalfa)	Amphipathic α helix Amphipathic α helix

^aCompiled from data in Bartels (1996) and Thomashow (1999).

composition with many charged or polar amino acids—only a few residues may account for >50% of the total amino acid complement, which makes them extremely hydrophilic. (iii) They are extremely stable proteins, which stay in solution in boiling water.

LEA proteins commonly show two structural motifs, random coils and amphipathic helices, sometimes in combination. Randomly coiled α helices are able to conform to the shape of the cellular structures they contact with. Because LEA proteins have many hydroxyl groups, those that have random coils in parts of their structure are thought to “solvate” cytosolic structures and prevent their crystallization. An amphipathic α helix, as the name implies, has a hydrophobic and a hydrophilic side. The hydrophobic face is thought to interact with lipids in membranes or with exposed hydrophobic patches of partially denatured proteins. The hydrophilic side of the helix, and other polar parts of the molecule, hydrogen bond with polar groups on macromolecules or other components in the cytosol or nucleosol. As a result, LEA proteins with amphipathic helices are thought to coat exposed hydrophobic surfaces with polar surfaces, which would prevent coagulation of membranes and macromolecules under dehydration.

The Em protein in wheat (LEA I group) and dehydrins (group II LEAs) are examples of proteins with random coils and amphipathic helices. The Em protein is more than 70% random coil and, under normal physiological conditions, is considerably more hydrated than most globular polypeptides. Dehydrins are characterized by several conserved domains, including a 15 amino acid lysine-rich sequence, known as the K segment (Fig. 16-20). The K segment occurs in tandemly repeated (up to 11) copies separated by ϕ segments, which are rich in polar amino acids. K segments are thought to form amphipathic helices.

Group III LEAs contain repeats of an 11 amino acid motif, which is also believed to form amphipathic helices. The helices are thought to form intramolecular bundles, which would present a hydrophilic surface on one side and a hydrophobic surface on the other. Because one of the effects of dehydration is increased salt or ionic content in the cell, it is possible that the hydrophilic, charged amino acids along one face bind ions, whereas the other face binds membranes. The magnitude of ion scavenging has been calculated: for mature embryo cells of cotton, the D7 protein represents about 4% of the nonorganellar cytosolic protein, enough to sequester >1.5 mM phosphate salts!

Many genes encode novel polypeptides that do not fall into any of the LEA groups but share some features of the LEA proteins: they are hydrophilic, many remain soluble upon boiling in dilute aqueous buffer, and many have relatively simple amino acid compositions, being composed largely of a few amino acids (e.g., CAS15 in alfalfa is rich in glutamate, histidine, and lysine, which account for 68% of the amino acid residues). Many of these polypeptides also contain regions of repeated amino acid sequences, predicted to form amphipathic α helices.

Evidence for a role of LEA and other hydrophilic proteins in stress tolerance has been obtained by overexpressing them transgenically. For example, rice plants transformed with the barley *HVA1* cDNA, encoding a LEA III protein, show an enhanced tolerance to drought, salinity, and freezing. Transgenic *Arabidopsis* plants expressing *COR15a*, which encodes a mature polypeptide COR15am resident in plastids, show increased freezing tolerance of chloroplasts *in situ*. They also show enhanced stability of

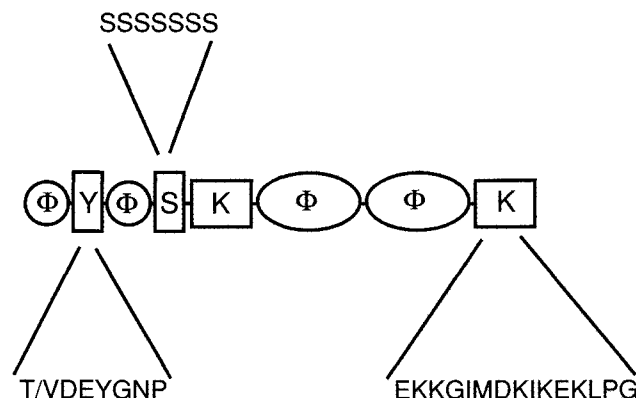


FIGURE 16-20 The common structural motif of dehydrins. The N-terminal Y segment is followed by a serine-rich tract (the S segment), which is followed by one to several copies of K segments, which are believed to form α helices. K segments are separated by other lesser conserved and usually repeated domains (the ϕ segments). From Close (1997).

isolated plasma membrane fractions against freeze-induced lesions *in vitro*. But the mechanism by which these proteins actually bring about enhanced tolerance against desiccation stress remains elusive. It is not known whether they bind water or prevent coagulation of membranes or macromolecules by forming a hydrophilic interphase, or sequester salts, *in vivo*. It is also not known whether they serve as osmolytes in osmotic adjustment and/or as osmo- or cryoprotectants, although it is thought that in the latter function they would be more efficient on a per molar basis than simple organic compounds such as sugars. Some of these proteins might also serve as AFPs.

2. Transporter proteins. Transporter proteins are membrane integral proteins (MIPs), which are involved in the movement of osmolytes, such as ions and organic molecules, or water across membranes. They include ATPases, pyrophosphatases, and aquaporins (water carrier proteins). [MIPs can be further divided into plasmalemma integral proteins (PIPs) and tonoplast integral proteins (TIPs).] Plasma membrane as well as tonoplast ATPase genes are induced by high salt stress. However, ATPases belong to multigene families, and which particular ATPases are induced under osmotic stress is not known. Water relations of a cell are affected not only by the concentration of the osmolytes, but also by the ease with which water molecules can move in and out of the cells or vacuoles. Aquaporins facilitate water movement by forming small pores across membranes. Aquaporin genes are reported to be both upregulated under water stress in pea and *Arabidopsis* and downregulated in the ice plant (*Mesembryanthemum crystallinum*), but the significance of such regulation is not clear.

3. Proteins with a role in preventing oxidative stress. Electron transport during photosynthesis, respiration or processes linked with plasmalemma inevitably results in a leakage of electrons, which are accepted by molecular oxygen. The result is the production of a highly reactive, superoxide anion radical (O_2^-), which, upon further reaction within the cell, can form hydrogen peroxide (H_2O_2), hydroxy radicals (OH^\cdot), and singlet oxygen. All are members of the highly reactive oxygen species (ROS), which are known to activate endonucleases with resultant damage to DNA, cause proteolytic cleavage of proteins, peroxidation of unsaturated fatty acids in membrane lipids, interfere with normal electron transport, and, in general, cause havoc with the cells.

Efficient removal of ROS is performed by superoxide dismutases (SODs) and a number of other antioxidants, which probably act in concert. SODs are metalloenzymes with Fe, Mn, or Cu/Zn at the active site, which catalyze the first step in scavenging the ROS, i.e., dismutation of the superoxide radical to hydrogen peroxide and molecular oxygen. Plants also produce a number of antioxidants, e.g., ascorbic peroxidase, glutathione, and α -tocopherol, which convert H_2O_2 to molecular oxygen.

In plants under dehydrative, or chilling stresses, the levels of ROS are enhanced manyfold. For instance, plasmalemmae of plant cells, under biotic or abiotic stresses, produce oxidative bursts of H_2O_2 . Such large amounts may be produced that the antioxidative defenses of the cell are temporarily overwhelmed. Thus, it is to be expected that the levels of enzymes such as SODs (or enzymes involved in the synthesis of other antioxidants) are activated on stress. A number of studies on SODs show that they are encoded by multigene families, with members showing compartmentation in cytoplasm, mitochondria, or plastids (the O_2^- radical is unable to cross membranes and must be deactivated at the site of production), and that members of the gene family are differentially expressed in response to ABA and high osmoticum.

4. Heat shock proteins (HSPs). Most proteins and enzymes are sensitive to heat inactivation and denaturation, a phenomenon that severely limits the survival of plants (and other organisms) at supra-optimal temperatures. Adaptive mechanisms exist, therefore, that protect cells from damaging effects of heat stress. Sublethal doses of heat stress induce a cellular response, known as the heat shock response, which consists of a temporary cessation of normal protein synthesis, and its replacement by enhanced synthesis of HSPs. Subsequently, normal cellular and physiological activities are resumed. Such resumption may involve the synthesis of new isoforms of enzymes that are tolerant to higher temperatures.

HSPs are a large group of highly conserved proteins. Several major groups are recognized, based on molecular weights [e.g., HSP100, HSP90, HSP70, HSP60, and the small molecular weight or smHSPs (10–30 kDa)]. Different HSPs probably serve different functions, as is suggested by the fact that, except for smHSPs and HSP100, all other HSPs are expressed at normal temperatures in moderate amounts. The incidence of smHSPs and HSP100 increases dramatically on heat shock.

HSP genes are expressed in plants not only under heat shock, but also under many other environmental stresses, drought, salinity, cold, flooding, and anoxia, heavy metal stress, and oxidative stress. It is

reasonable to assume, therefore, that HSPs are synthesized either to protect the plant from damage or to repair the damage caused by stress. It was mentioned earlier that one type of stress can induce protection against another stress, i.e., offer cross-protection. In a seemingly paradoxical way, heat shock can offer protection against chilling injury. One study showed that mature, green tomato fruits kept at 38°C for a few days and then transferred to storage at 2°C were able to tolerate the cold temperature, whereas control fruits kept at room temperature developed chilling injuries. It was shown subsequently that heated fruits developed HSPs, which were maintained if fruits were transferred to 2°C, but were lost at room temperature. Similar high temperature-induced protection against chilling is known for vegetative tissues and seeds as well.

How HSPs protect cellular machinery is still unknown, but there is increasing evidence that they do so by acting as "chaperons." Molecular chaperons are defined by their capacity to recognize and to bind substrate proteins that are in an unstable, inactive state. Under stress of any kind, the number of cellular proteins/enzymes that are in an unstable or inactivated/denatured state increases dramatically. These proteins have to be recognized and, if possible, salvaged; if not, degraded and broken down. How HSPs are able to do these functions is not clear. Some data suggest that smHSPs in plants aggregate to form large multimeric protein complexes ranging from 200 to 800 kDa, and it is these multimers that are physiologically active. Some have been reported to refold denatured proteins in an ATP-independent manner.

5. Defense-related proteins. Enzymes involved in defenses against pests and herbivores (e.g., proteinase inhibitors, phenylalanine ammonia lyase, chalcone synthase) and bacterial and fungal pathogens (e.g., osmotins, chitinases, glucanases) are accumulated in responses to osmotic or cold stress. Genes encoding these proteins belong to multigene families, and details as to which specific gene is expressed under a particular stress are not clear.

6. Other proteins. Alcohol dehydrogenase (ADH) is one of the enzymes used in anaerobic breakdown of pyruvate. *ADH* genes are expressed under anoxia due to flooding or under cold stress. Lipid transfer proteins (LTPs) transfer lipids between membranes *in vitro*, e.g., between artificial membrane vesicles (liposomes) and mitochondria. By analogy, they are believed to serve a similar function *in vivo*. *LTP* genes are expressed in epidermal cells and are believed to be responsible for the transfer of lipids to the cell exterior for the synthesis of cutin and wax. *LTP* genes are also induced under dehydrative stress, although the exact connection is not obvious.

6.2. The Same Gene May Be Upregulated by Two or More Stresses as Well as ABA

The expression of several genes is upregulated by two or more stress factors as well as ABA. For instance, *RD29A* in *Arabidopsis* (also known as *COR78* and *LTI78*, see Table 16-2) is induced by drought, salinity, low temperature, or ABA. Other genes regulated by two or more stresses and ABA include *COR6.6/KIN1*, *COR47*, and *LTI30* in *Arabidopsis* and *HVA1* in barley. Several genes occur as members of a family, and different members of the same gene family may be differentially regulated by different stresses. *COR15a* and *COR15b* are members of a gene family that occur as a tandem pair in the *Arabidopsis* genome. Both are induced by low temperature, as well as application of exogenous ABA to unstressed plants. However, *COR15a* is also upregulated by drought, whereas *COR15b* is not. Because different members of the

gene family encode different isoforms of the same protein, it could be that isoforms induced by several stresses serve a more general function, whereas others with restricted expression are specific to a particular stress. The differential regulation of members of a gene family is a common phenomenon in plant development.

Although many genes are expressed under two or more stresses, the magnitude of response to the two stresses may not be the same. In fact, many genes that are responsive to both low temperature and drought respond more to one type of stress than to the other. A comparison of two *Arabidopsis* genes, *RAB18*, which responds primarily to drought, and *LTI78*, which responds primarily to low temperature, is shown in Fig. 16-21. It is clear that *RAB18* responds mostly to drought and very little to low temperature, whereas *LTI78* responds only moderately to drought. The response of *LTI78* to ABA is also moderate, whereas that of *RAB18* is substantial.

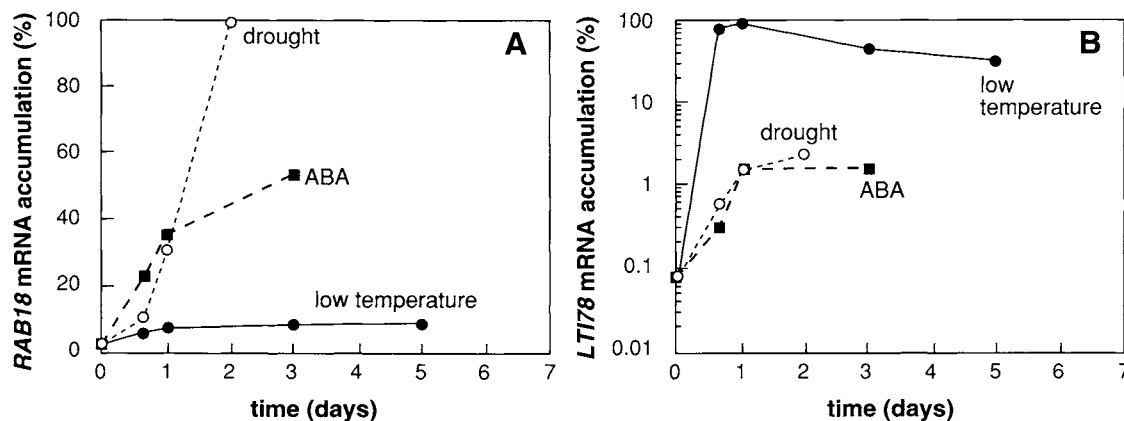


FIGURE 16-21 Accumulation of *RAB18* and *LTI78* mRNAs of *Arabidopsis thaliana*. RNA was extracted from plants grown under low temperature (4°C day/2°C night) or drought conditions (60–70% relative humidity) or after ABA (60 μ M) treatment for days indicated. 32 P-labeled *RAB18* or *LTI78* probes were used for Northern blots, and radioactivity on the membrane was quantified using a Phosphorimage scanner. Note that the X axis for B showing *LTI78* mRNA accumulations is a logarithmic scale. Adapted from Lång et al. (1994).

6.3. Stress-Related Gene Expression Can Be Induced Independently of ABA

For genes that are upregulated by a particular stress and also by exogenous ABA under nonstress conditions, it could be assumed that the plant response to stress is mediated by ABA. However, is the gene expression mediated *via* ABA (endogenous) or can it be regulated independently by the stress? Use of wild-type (WT) plants and ABA synthesis and ABA insensitive mutants provides a means to separate ABA-induced vs a stress-induced gene expression. For accurate comparison, it is essential that the WT and mutant plants come from the same genetic background. Fortunately, such mutants are available for some species, notably *A. thaliana*.

Table 16-4 shows the expression of six genes from *Arabidopsis* in wild-type plants and in ABA synthesis (*aba1*) and ABA-insensitive (*abi1*) mutants. All are expressed from low to high transcript levels in response to cold acclimation, drought, or ABA (without water stress). Data from low temperature treatment reveal that the cold-induced expression of *LTI140*, *LTI30*, *LTI45*, and *LTI78* is essentially normal in plants carrying the *aba1* mutation. Moreover, the *abi1* mutation, while abolishing the ABA-induced accumulation of transcripts of these genes, has little or no effect on cold-induced accumulation of their transcripts. Thus, it appears that cold temperature and ABA independently regulate the expression of these genes.

A comparison of transcript accumulation under drought and applied ABA reveals that in drought-stressed *aba1*, these genes show little or no expression,

but in the presence of exogenous ABA they are expressed almost to the same extent as in the WT. The ABA-insensitive mutant *abi1* is unable to express any of them in the presence of exogenous ABA, but noticeably two genes are expressed to a moderate extent in drought-stressed *abi1* mutants. These data from drought-stressed *abi1* mutants also suggest that at least some genes are regulated by drought independently of endogenous ABA.

Table 16-4 also provides data for *RAB18* and *ADH* genes, which are comparable to and confirm data for *RAB18* and *LTI78*, respectively, in Fig. 16-22.

6.4. Stress-Signaling and ABA-Signaling Pathways May Intersect with Each Other

How ABA, drought, and low temperature independently regulate the expression of some genes requires an analysis of promoter sequences and the identification of *cis*-acting elements and transcription factors that bind to them. These topics are discussed in Chapter 23, along with ABA-induced gene expression. However, it should be mentioned here that it is likely that signaling pathways from osmotic and cold stresses and from ABA interact with each other or converge at some point. Support for this proposition comes from some *Arabidopsis* mutants that have been generated using the technique of targeted genetics. *RD29A* is an *Arabidopsis* gene expressed in response to multiple stimuli, cold acclimation, drought, high salinity, and ABA. Jian-Kang Zhu at the University of Arizona, Tucson, and associates used a chimeric gene construct containing the *RD29A* promoter and the

TABLE 16-4 Effects of Water Stress (Drought) and ABA on Expression of Six Genes in *Arabidopsis thaliana* Wild Type (WT, Landsberg erecta), ABA-deficient *aba1* Mutant, and ABA-Insensitive *abi1* Mutant

Gene	Treatment								
	Low temperature			Drought			ABA		
	WT	<i>aba1</i>	<i>abi1</i>	WT	<i>aba1</i>	<i>abi1</i>	WT	<i>aba1</i>	<i>abi1</i>
<i>LTI140</i>	+++	+++	+++	+++	nk ^a	++	+++	++	0
<i>LTI30</i>	+++	+++	+++	++	+	++	++	+++	0
<i>LTI45</i>	++	++	+++	+	0	0	+	++	0
<i>LTI78</i>	+++	+++	+++	+	0	+	+	+	+
<i>RAB18</i>	+	0	+/-	+++	0	+/-	+++	+++	+/-
<i>ADH</i>	+++	++	++	+	0	0	++	+	0

^ank, Not known. Drought-stressed plants were withheld water. Plants supplied with ABA (10^{-5} M) were kept well watered. Adapted with permission from Hughes and Dunn (1996), ©1996 Oxford University Press.

coding sequence of a firefly *LUCIFERASE* (*LUC*) reporter gene, as shown below, to stably transform *Arabidopsis* plants (LB and RB refer to left and right borders of the T-DNA construct).

LB >—————< RB
RD29A promoter *LUC* coding sequence NPT - II

As a result, these plants, when subjected to cold, salt, drought, or ABA treatment, emitted feeble light signals, which could be monitored by video-imaging enhancement. Seeds from transgenic plants homozygous for the transgene were mutagenized by ethylmethanesulfonate, and the M₂ seedlings were screened for

mutants with altered bioluminescence. A large number of *cos*, *los*, or *hos* (for constitutive, low, or high expression of osmotically responsive genes) mutants were identified. Based on the responses of *los* and *hos* mutants to one or a combination of stress and ABA signals, Zhu and colleagues have proposed a model for stress signaling in higher plants, where the ABA-dependent and stress-dependent signaling pathways cross-talk with each other and converge at some point to activate stress gene expression. In addition, some *hos* mutants show enhanced expression of *RD29A* and several other cold-regulated genes under low temperature, but not under ABA or high salt treatment (e.g., *hos1* and *hos2*), whereas others show enhanced

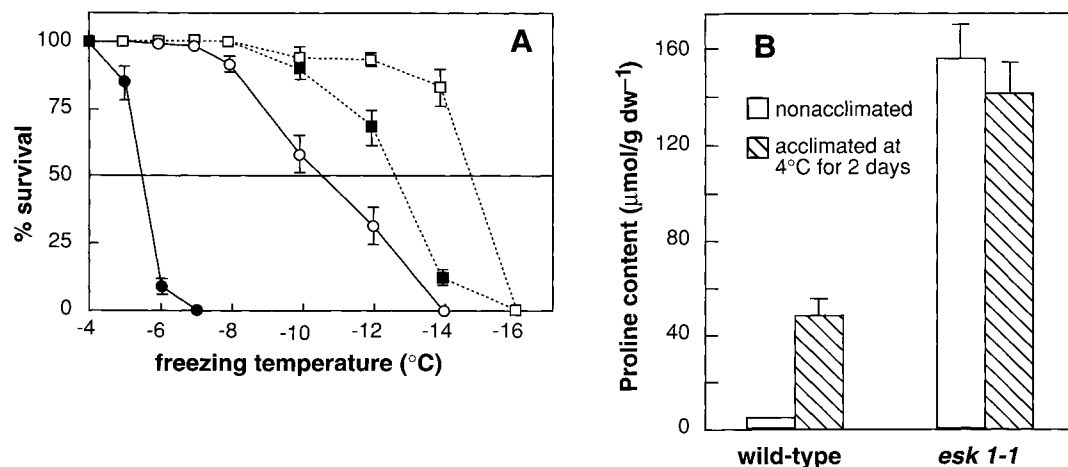


FIGURE 16-22 Freezing survival and proline content of wild-type and *esk1* mutants of *Arabidopsis*. (A) Percentage survival of wild-type (closed symbols) and *esk1* (open symbols) plants. Solid lines, nonacclimated; dotted lines, after acclimation at 4°C for 2 days. (B) Proline accumulation in wild-type and *esk1* plants without acclimation and after cold acclimation at 4°C for 2 days. From Xin and Browse (1998).

expression under ABA and osmotic stress, but not when exposed to low temperature (e.g., *hos5*).

In summary, tolerance to any one stress requires the expression of many genes. Also, the same gene may be expressed under two or more stresses, thus providing the molecular basis for cross protection. Many stress-related genes are also induced by ABA. Data using an ABA-insensitive mutant indicate that both drought- and low temperature-responsive genes may be regulated independently of ABA or may form part of an interconnected network in which signals from multiple stresses and from ABA converge.

7. ISOLATION OF STRESS-RELATED GENES USING MUTATIONAL ANALYSIS

Stress-related responses are quantitatively inherited traits that are regulated by multiple genes, and there have been relatively few attempts to identify cold, salt, or drought-regulated genes by mutational analysis. Nonetheless, screens designed to select mutants that are affected globally (not in a specific trait) in their capacities to tolerate freezing or high salinity have yielded some mutants in *Arabidopsis* (Table 16-5).

sfr Mutants do not develop the same amount of freezing tolerance after cold acclimation as the wild type. They seem to be affected in different aspects of freezing tolerance. For instance, some show a reduced accumulation of sugars, anthocyanin, and lowered levels of 18:1 and 18:2 fatty acids, whereas others, (e.g., *sfr6*) are unable to express typical cold-responsive genes such as *KIN1*, *COR15A*, and *LTI78*. The *eskimo1* (*esk1*) mutant displays constitutive cold acclimation and accumulates much higher amounts of proline (~ 15-fold higher) and total sugars than the wild type (Fig. 16-22). Expression of the *RAB18* gene is

elevated about three-fold, but significantly, the expression of several cold-responsive genes is normal in the *esk1* mutant.

Several mutants and mutant lines have been isolated that tolerate or whose seeds can germinate under salt concentrations that are completely inhibitory to the wild-type ancestors (e.g., *san* mutants can germinate under 250 mM NaCl). The seeds of *san5* in particular display tolerance to salts (NaCl, KCl) and mannitol, as well as insensitivity to ABA in germination. The *san5* is allelic to *abi4*, one of the ABA-insensitive mutants of *Arabidopsis*. The *ABI4* gene encodes a transcription factor involved in ABA signaling, and the deduced polypeptide sequence of *san5* lacks the DNA-binding domain of *ABI4*.

The salt overly sensitive (*sos*) mutants are covered in the next section.

Studies on stress-related mutations are still in a preliminary stage, but they confirm that regulation of stress tolerance occurs at multiple levels because while some traits are affected in these mutants, others are not. The mutations seem to be in regulatory genes encoding transcription factors, which directly or indirectly affect the expression of some, not all, genes associated with cold acclimation or salinity tolerance. Some, such as *ESK1*, may regulate negatively, whereas others (e.g., *SFR4*) regulate positively. Moreover, *SAN5* seems to reinforce the link between stress signaling and signaling by ABA.

8. IMPROVING STRESS TOLERANCE OF CROP PLANTS

Extremes of temperature and salinity impose strong limitations to lands that are suitable for agriculture and the productivity of crop plants. Large tracts of land experience a short growing season and freezing temperatures in winter, whereas many others are arid

TABLE 16-5 Some *Arabidopsis* Mutants Affected in Their Tolerance to Freezing or High Salinity

Mutant	Phenotype	Ref.
<i>sfr</i> (for sensitive to freezing)	Defective in achieving freezing tolerance	Warren <i>et al.</i> (1996)
<i>esk1</i> (for eskimo1)	Constitutively cold acclimated	Xin and Browse (1998)
<i>sos</i> (for salt overly sensitive)	Hypersensitive to salt	Wu <i>et al.</i> (1996)
<i>sañ</i> (for salobreño, Spanish for salty land)	Seeds germinate at high NaCl concentration (250 mM)	Quesada <i>et al.</i> (2000)

or semiarid or are naturally saline. Moreover, irrigation water from wells or rivers, or use of chemical fertilizers, adds minerals and salts to irrigated fields. Due to evaporation from soil and transpiration from plants, salts gradually accumulate and increase the salinity of the soil. A large percentage of agricultural lands around the world are so threatened with increasing salinity.

Improvements in stress tolerance by sensitive crop plants are difficult to accomplish by plant-breeding techniques for two reasons. First, stress tolerance traits are quantitative in nature and controlled by multiple genes, and improving tolerance to freezing or osmotic stress by breeding has proven a formidable task. For example, traditional plant-breeding efforts have improved the freezing tolerance of wheat varieties only marginally since the early 1900s. This is in contrast to tolerance (resistance) to biotic stresses (e.g., pathogens), which are often regulated at single gene level. Second, breeding with more distant, wild relatives, which are tolerant to a stress (e.g., salinity), runs the risk of introducing undesirable traits, such as poor palatability, poor processing properties, and even toxins.

Genetic engineering is attractive because it avoids the problem of loss of important agronomic traits, as only a single (or a few) tolerance gene is introduced into the target crop by each transformation event. The problem with genetic engineering has been the lack of good tolerance genes. For example, in the last decade, many attempts have been made to enhance the tolerance of crop plants to freezing or salinity by the introduction of genes encoding enzymes for the synthesis of osmolytes, such as proline, betaine (see Section 5), mannitol, and fructans, in crop plants (e.g., tobacco, rice, potato). Transgenic rice plants expressing a LEA protein from barley (HVA1) have been produced. Alfalfa plants have been transformed with cDNAs encoding antioxidants, such as a superoxide dismutase. Such transformations in most cases show improved tolerance; nonetheless, the level of osmolyte or product accumulated is not sufficient to confer stress tolerance under field conditions. Accordingly, new methods are being tried. Transformations involving regulatory genes that encode transcription factors are a possibility. For example, transformation of *Arabidopsis* plants with a gene encoding a transcription factor that regulates the expression of many cold-regulated as well as water stress-regulated genes results in the expression of several *COR* genes without cold acclimation and confers a better freezing tolerance to plants than transformation with a single *COR* gene.

Stress tolerance depends on complex interactions between several types of gene products. In most cases, these interactions are unknown. An example of such an interaction is provided by the *sos* (for salt overly sensitive) mutants of *Arabidopsis*. In contrast to mutants that tolerate high salt concentration, *sos* mutants show inhibition of root growth at concentrations of NaCl that do not inhibit root growth in the wild type. For example, the *sos1* mutant shows inhibition at 20-fold lower NaCl content than the wild type. At these low concentrations of salt, osmotic stress is not involved; hence, some other explanation must account for the hypersensitivity. K^+ ions are taken up by roots by two uptake mechanisms: a high-affinity mechanism, which is operative in soils with a low potassium content, and a low-affinity mechanism, which is operative when the potassium concentration is not limiting. Because the soil potassium content is usually low ($> 1 \text{ mM } K^+$), the high-affinity mechanism is thought to play the predominant role in plant potassium nutrition. High concentrations of Na^+ in the soil disrupt potassium uptake, specifically the high-affinity mechanism. Thus, *sos* mutants show reduced growth not because of osmotic stress, but because they cannot pick up enough potassium. Several *sos* mutants are known.

For *sos3* mutant, the enhanced sensitivity to salt is due to an interaction between the K^+ uptake by roots and the effect of Ca^{2+} ions on that uptake—the presence of 3 mM calcium ions relieves this inhibition and restores *sos* mutants to the wild phenotype (Fig. 16-23). The *SOS3* locus has been cloned and, not unexpectedly, encodes a calcium-binding protein with a possible function in signaling.

Among other approaches is the genetic approach of crossing cultivars with known loci for quantitatively inherited traits. These loci, known as **quantitative trait loci** (QTL), relate performance and yield of a cultivar (e.g., of wheat or barley) to tolerance to a specific stress, drought, salinity, or low temperature. Regions of chromosomes that carry genes that improve stress tolerance can be mapped and crosses made; e.g., between Chinese spring wheat and cultivated wheat, which differ in their degree of salinity tolerance. This approach allows a selection of phenotype with desirable physiological traits, without necessarily a knowledge of the genotype. However, a wide application of this approach is limited by the need to assess not only the parents, but also large numbers of individuals and families in segregating generations. Moreover, genotypic information is required in the form of markers needed for the selection of QTLs or of direct knowledge of the genes.

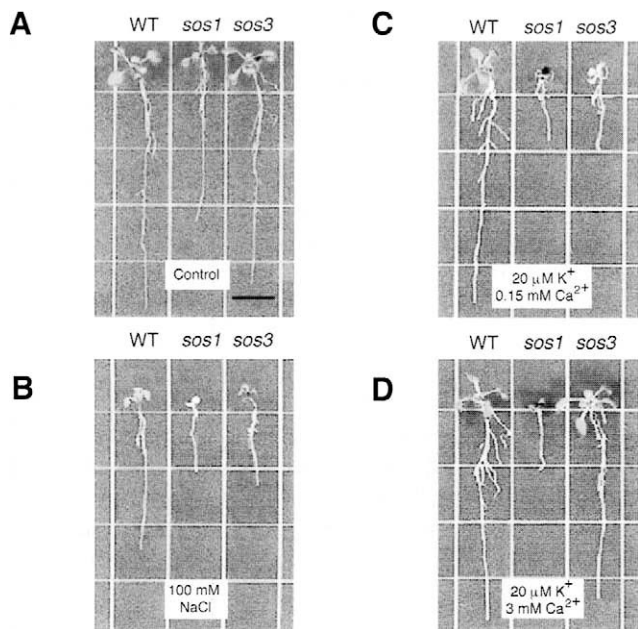


FIGURE 16-23 Representative plants of wild-type (WT), *sos1*, and *sos3* mutants exposed to high salt (100 mM NaCl) or low K^+ ($20 \mu M K^+$) stresses. Seedlings were grown for 4 days on nutrient agar and were then transferred to new medium with only nutrients (A, control), nutrients plus 100 mM NaCl (B), and nutrients plus $20 \mu M K^+$ with $0.15 mM Ca^{2+}$ (C) or $3 mM Ca^{2+}$ (D) for 7 days. The growth of both *sos1* and *sos3* was inhibited by either NaCl (B) or low K^+ (C); however, the growth of *sos3* on low K^+ was restored by increased Ca^{2+} (D). Bar: 1 cm. From Liu and Zhu (1997).

9. CHAPTER SUMMARY

Tolerance to biotic and abiotic stresses is vitally important to the survival of plants. This chapter considered tolerance to three types of abiotic stresses; drought, salinity, and cold temperature. These stresses elicit different responses, but because they all cause desiccation of cells, they also elicit several common responses. This is the basis for the phenomenon of cross-protection: exposure to one stress provides protection against other stresses. Plants respond to these stresses essentially in three ways: conserving water by closing stomata; raising the intracellular levels of low molecular weight organic compounds; and synthesizing various types of proteins. ABA plays a central role in mediating these responses. In mesophytic plants subjected to water deficit, ABA synthesized in the root and transported via the xylem or synthesized in the shoot accumulates in the leaf apoplast, causing stomatal closure. On rewatering, the apoplastic ABA is taken up rapidly by the symplast and metabolized to inactive products. A rise in ABA levels in the shoot may also inhibit photosynthesis and shoot growth, but the causality is less clear. Plants living in temperate

and subpolar regions are exposed to freezing temperatures during the winter. They cold acclimate during the fall months, a process that can be duplicated in the laboratory or a greenhouse by exposing plants to chilling temperatures. Part of this acclimation involves a transient rise in endogenous ABA content, a change in membrane lipid composition, including an increase in proportions of polyunsaturated fatty acids, a synthesis of antifreeze proteins, which in some plants occur apoplastically, and an enhanced capacity for supercooling. Other responses to freezing stress, e.g., synthesis of osmolytes and proteins, are shared responses with drought and/or salinity stress. A variety of low molecular weight N_2 -containing compounds and/or carbohydrates (sugars, sugar alcohols, and oligosaccharides) are accumulated that help in osmotic adjustment and also protect membranes and macromolecules against dehydration damage. A number of different types of proteins are synthesized; proteins that are thought to keep membranes and macromolecules solvated, bind excess salt, scavenge dangerous and reactive oxygen-free radicals, recognize and remove proteins that have been inactivated due to stress, and serve protective functions against pests and pathogens. ABA is involved in triggering the expression of many genes encoding these proteins. Some of these genes are also induced by water deficit or low temperature independently of ABA, although it is also thought that ABA- and stress-signaling pathways interact and converge before gene expression. Some genetic mutants in *Arabidopsis* have been isolated in which the responses to salinity or cold acclimation are affected. The mutations appear to be in genes encoding transcription factors and confirm that the regulation of stress responses occurs at multiple levels. Attempts to improve stress tolerance of crop plants by breeding techniques or by molecular genetic means have only achieved limited success to date, but new approaches are being tried. One of the major impediments is lack of knowledge of the complex interactions between different factors that together constitute tolerance to an abiotic stress.

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Fruit Development and Ripening

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1. FRUIT DEVELOPMENT OCCURS IN REGULATED STEPS

Fruit is the structure that arises from an ovary (or several ovaries and, in some cases, associated floral parts) after fertilization and supports the developing seeds. It is an evolutionary adaptation unique to flowering plants and is designed to aid in the dispersal of seeds by various agencies, including animals, wind, and water. Plants spend considerable amounts of energy in developing fruits, especially fleshy fruits, massive amounts of food materials are imported into these structures from the rest of the plant and, thus, it is to be expected that fruit development would be controlled at discrete steps.

2. FRUITS COME IN AN IMMENSE VARIETY

There is much variation among flowering plants in the manner in which fruits arise, whether they arise from single or multiple but fused ovaries of a single flower (simple fruits), from several single ovaries of a single flower (aggregate fruits), or from ovaries of several flowers in an inflorescence (multiple fruits). Simple fruits can be fleshy (e.g., tomato, apple, melon, avocado) or dry (e.g., poppy). Aggregate (e.g., strawberry, blackberry) and multiple fruits (e.g., pineapple) are usually fleshy and, in some of these fruits, associated structures, such as the receptacle bearing the ovaries, become part of the fruit. Fruits also vary in

their final shapes and sizes (cf. samara of maple, acorn of oak, fleshy fruits of elderberry, black currant, pear, or zucchini) and the changes that occur during ripening (cf. cucumber, papaya, watermelon, or orange).

Although there is an immense variety of fruits, much of our information on the physiology and biochemistry of fruit growth and development comes from a study of fleshy fruits, although some aspects, such as parthenocarp, have also utilized dry fruits. In the following account, the emphasis is on fleshy fruits.

3. STAGES IN FRUIT DEVELOPMENT

In most species, fruit growth can be represented by a sigmoidal curve or a double sigmoidal curve with a second burst of growth during the ripening period. These two patterns can occur in the same species, as shown for tomato cultivars Pik-Red and Ailsa Craig (Fig. 17-1).

Physiologically and biochemically, fruit development can be divided into four phases, which, although continuous, are separated on the basis of the major activities (Fig. 17-2). Phase I includes ovary development in the flower, and following

anthesis (i.e., rupture of anthers to release mature pollen), a decision to abort or proceed with further development. Phase II involves a period of rapid cell divisions. Phase III is the period of most rapid growth, when cell divisions more or less cease, and growth is almost exclusively by cell enlargement. In this phase, food reserves are accumulated and most fruits attain their final shape and size before the onset of ripening in phase IV. In some others, as mentioned earlier, there may be another burst of growth during the ripening period. Also, in some fruits, such as avocado, cell divisions may continue well into Phase III.

While the last phase, that of fruit ripening, has been well studied because of its importance in fruit harvest and storage, there is still relatively little information on the first three phases.

4. GROWTH AND DEVELOPMENT TO MATURE STAGE

Three hormones, auxin, gibberellins (GAs), and cytokinins (CKs), are involved in the early stages of fruit growth, especially phases I and II, although their

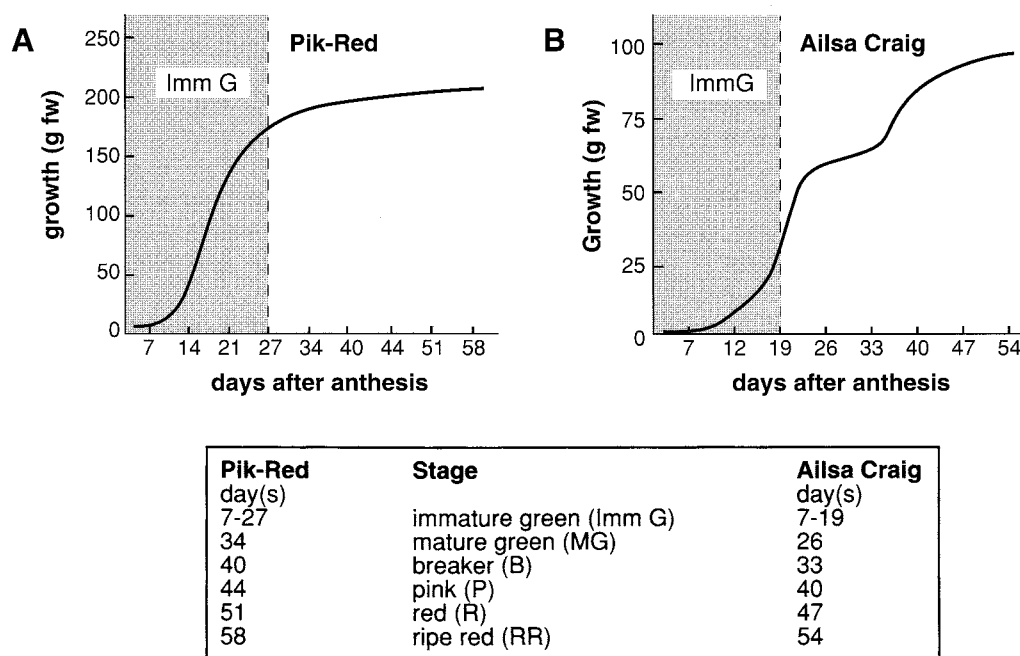


FIGURE 17-1 Tomato (*Lycopersicon esculentum*) fruit growth during development and ripening. (A) Cultivar Pik-Red shows a typical sigmoidal curve with most growth finished before ripening. (B) Cultivar Ailsa Craig shows a double sigmoidal curve with a second spurt of growth during ripening. Stages in growth and ripening (e.g., breaker, pink, ...) and days to attain them are indicated. Adapted from Buta and Spaulding (1994).

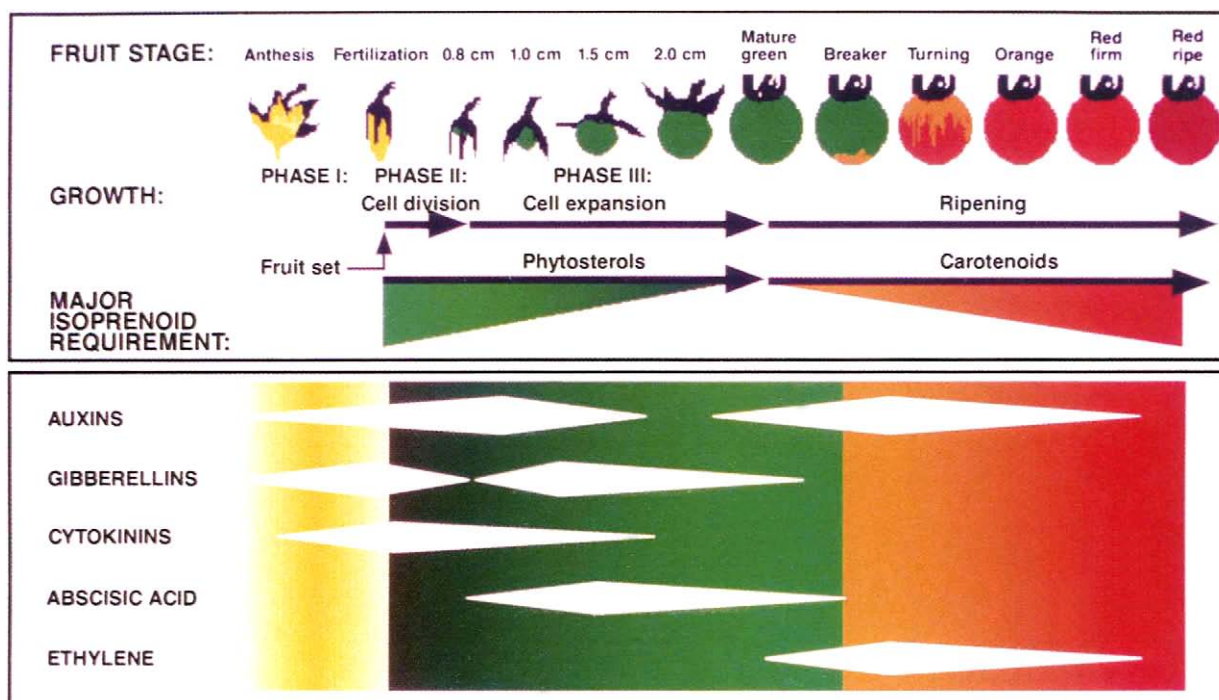


FIGURE 17-2 Fruit development and ripening in tomato (*Lycopersicon esculentum*), a simple fleshy fruit (a berry), that is often taken as a model to illustrate fruit development and ripening. Phases I–III represent periods of fruit set and growth; phase IV represents ripening. Changes in levels of different hormones at different stages are indicated by white diamonds. Among the major isoprenoids, sterols predominate during periods of rapid cell division and growth, whereas carotenoids predominate during fruit ripening. Modified from Gillaspy *et al.* (1993).

exact roles and interrelations among them are uncertain and unclear. In phase I, the source of these hormones is clearly maternal tissues, but in phases II and III, which coincide with embryo growth and maturation, respectively, the source of the hormones is debatable. The fruit is a maternal tissue, and these hormones could be synthesized *in situ* or translocated from other parts of the plant via the phloem stream. There are also instances where fruit growth is affected by auxins and/or cytokinins produced by the embryo and endosperm.

4.1. Pollination and “Fruit Set”

Growth of the ovary by cell division and cell expansion continues to sexual maturity of the flower, but then stops at the time of anthesis or shortly before pollination. The decision to resume growth is taken only if pollination occurs (but see Section 4.3). Ovaries remain receptive to pollen for some time after anthesis, which varies with species, but if they are still unpollinated to the end of the period, they undergo senescence and abscission. “Retention” of the ovary under the stimulus of pollination is known as setting fruit, or

“fruit set” (Fig. 17-3). Bees and other pollinators play an important role in orchards in providing this stimulus for fruit set.

For fruit set to occur, pollination is necessary, but fertilization is not. For instance, fruit set can be accomplished by the placement of foreign, but compatible, pollen on the stigma or by application of an aqueous pollen extract to the stigma or ovary. In both cases, fertilization does not occur.

Pollen extracts are believed to be rich in auxin, and pollen extracts can be replaced by auxin, especially in cultivars that are amenable to parthenocarpy (see below). It appears that pollination either provides auxin to the young ovary or, alternatively, provides the stimulus for the young ovary to synthesize its own endogenous auxin, which, in turn, promotes its growth and also inhibits its abscission.

Pollen grains of many species are also rich in brassinosteroids (BRs, see Chapter 9), but the effect of BRs on fruit set has not been investigated.

Although pollination causes fruit set and some growth of the young fruit, a second stimulus in the form of fertilization is needed to provide an impetus for continued growth under phase II. Fertilization is

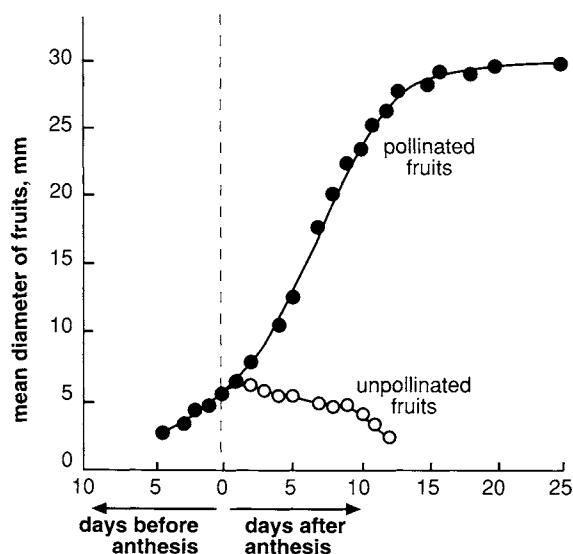


FIGURE 17-3 Growth curves of ovary of *Cucumis anguria*. Growth in pollinated ovaries shows a typical sigmoid growth curve, whereas unpollinated ovaries “shrive” and ultimately drop off. From Wareing and Phillips (1981).

not the only signal, however, that affects fruit growth or retention. Competition among different metabolic sinks for photoassimilate and resource allocation at the whole plant level are involved. For instance, decapitation (removal of actively growing apical tissues) promotes the growth of parthenogenetic pea pods. “Thinning,” or fruit drop, occurs despite successful fertilization and embryo formation and determines what proportion of the fruit crop is to be retained.

4.2. Fertilization and Further Growth

Phases II and III are marked by periods of rapid cell division and rapid cell expansion, respectively. These periods coincide with the growth of the embryo and

endosperm, and it has been suggested that auxins and cytokinins produced by the embryo and endosperm (see Chapter 18) have a role in fruit growth.

How does one demonstrate that a fruit being retained on the plant and growing is receiving its stimulus from the seed? One way is to look at the number of fully developed seeds and the final fruit size. That seeds influence fruit growth was shown very elegantly in the 1950s by Nitsch. Strawberry is an “aggregate” fruit, i.e., a fruit formed from several single carpels in a flower. Each carpel has a single seed and produces what is known as an “achene,” a joint structure of the seed and the surrounding ovary wall. Achenes are borne on the receptacle, which forms the fleshy fruit. Nitsch removed varying numbers of developing achenes from young fruit of strawberries and showed a strong correlation between the number of achenes left and the fruit growth around them (Figs. 17-4A and 17-4B). Similar positive correlations between the number of seeds and fruit growth have been shown for other fruits (e.g., tomato, grape, black currant, apple, pea pod).

Nitsch also showed that an exogenous application of β -naphthoxyacetic acid, a synthetic auxin, to the deached receptacle could replace the stimulus of fertilization for fruit growth (see Fig. 17-4C), which suggested that young seeds with developing embryo and endosperms could be a rich source of auxin and that this auxin was the trigger for continued growth of the fruit.

Measurements of endogenous IAA in developing fruits of tomato, using gas chromatography-mass spectrometry, show highest levels at the earliest time of sampling (7 days). The levels then drop steadily and then rise again to a lesser plateau during early stages of ripening (Fig. 17-5). Similar data have been reported for several fruits, including tomato, using bioassays and radioimmunoassay (see also Fig. 17-2).

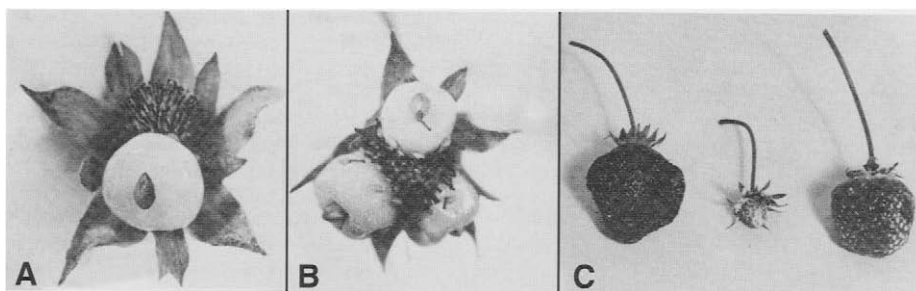


FIGURE 17-4 Strawberry fruit growth and its relation to number of achenes and exogenous auxin. (A and B) Removal of all but one or three achenes at a young stage led to fruit growth only around the remaining achenes. (C) Three strawberries of the same age: (left) control; (middle), all achenes removed and receptacle smeared with lanolin paste; and (right) all achenes removed and receptacle smeared with lanolin paste containing 100 ppm of the auxin β -naphthoxyacetic acid. From Nitsch (1950).

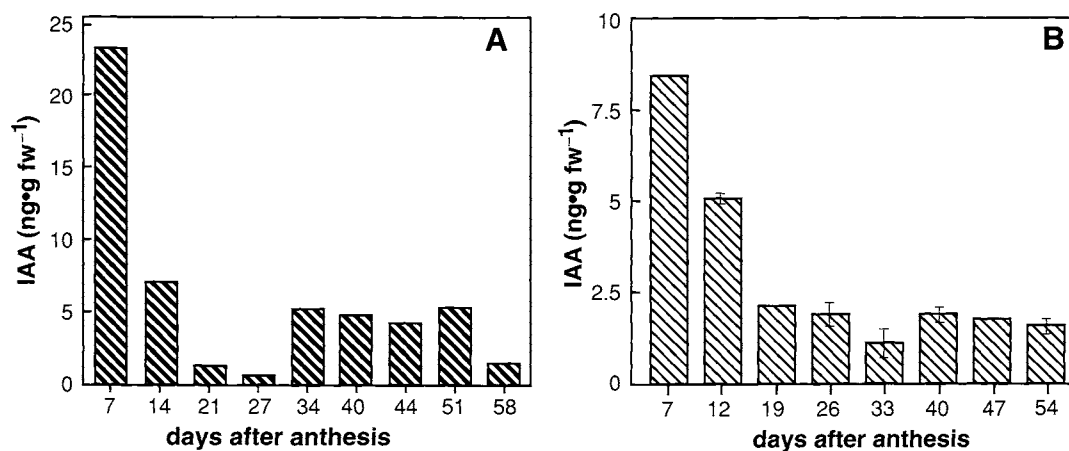


FIGURE 17-5 Endogenous IAA levels in tomato during fruit growth and ripening. Identification and quantitation were done using GC-MS. (A) Pik-Red, single sample analysis except for duplicate sample analyses at days 14 and 44 where average is shown. (B) Ailsa Craig, duplicate sample analysis. From Buta and Spaulding (1994).

In addition to auxin, cytokinins and gibberellins also play a role in growth of the young fruit. Cytokinin levels, especially zeatin and zeatin riboside, are high at the earliest stages of growth in several fruits (apple, tomato, orange) and correlate with the period of maximal cell division activity. In kiwi (*Actinidia deliciosa*) fruit, the concentration of total cytokinins was high at the earliest time after anthesis, dropped to low levels, and rose again before harvest 175 days after anthesis (Fig. 17-6). Data for zeatin and isopentenyladenine were generally similar.

The number of cells in the mature, unfertilized ovary and, subsequently, in early fruit growth is a critical parameter for the eventual fruit size. Thus, a larger size mutant of tomato has a larger number of cells than the smaller, "normal" fruit. That cytokinins play an important role in regulating cell divisions in the young fruit is shown by a comparison of cell numbers in fruits that are of different sizes in a single plant. The cultivar Hass of avocado (*Persea americana*) produces two populations of fruits, one significantly smaller than the other (normal) (Fig. 17-7A). The cell size in the two phenotypes is the same, but the smaller phenotype results from a reduced number of cell divisions, which, in turn, is related to a reduced cytokinin/abscisic acid (ABA) ratio in the smaller compared to the normal phenotype (Fig. 17-7B). The deficiency is corrected and the fruit size is restored to normal size by an exogenous application of a cytokinin, isopentenyladenine (iP), in phase I. Significantly, the deficiency is not corrected by application of GA₃.

Studies correlating gibberellin content with fruit growth are relatively few. In *Arabidopsis thaliana*, fruit (silique) growth requires endogenous GA

because it is impaired in the GA-deficient mutant, *gal1*, a defect that is restored to normal by an exogenous application of GA. In garden pea, the maximal growth rate of the pod is correlated with maximal GA₁ content. A similar correlation has been shown for gibberellin A₃ content and seed growth.

4.3. Parthenocarpic Fruits

Many species and cultivars produce fruit that either lack seeds or have no viable seeds. The production of such seedless fruits is known as parthenocarpy and is

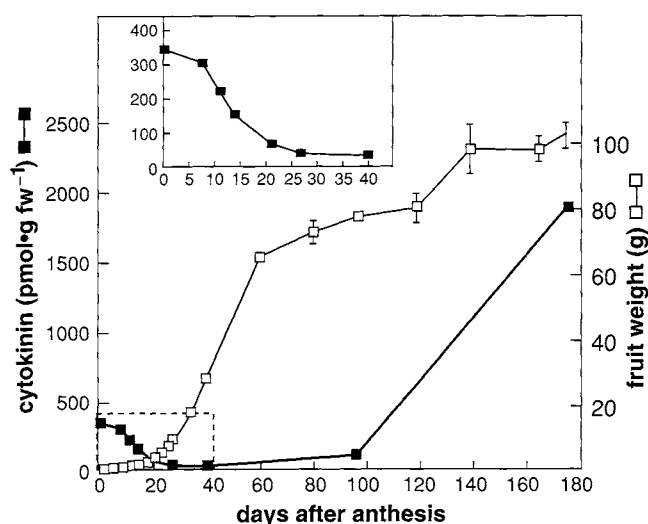


FIGURE 17-6 Fresh weight and cytokinin content of kiwi fruit harvested at different stages of growth. (Inset) An expanded view of the changes in CK concentration during early fruit development. From Lewis *et al.* (1996).

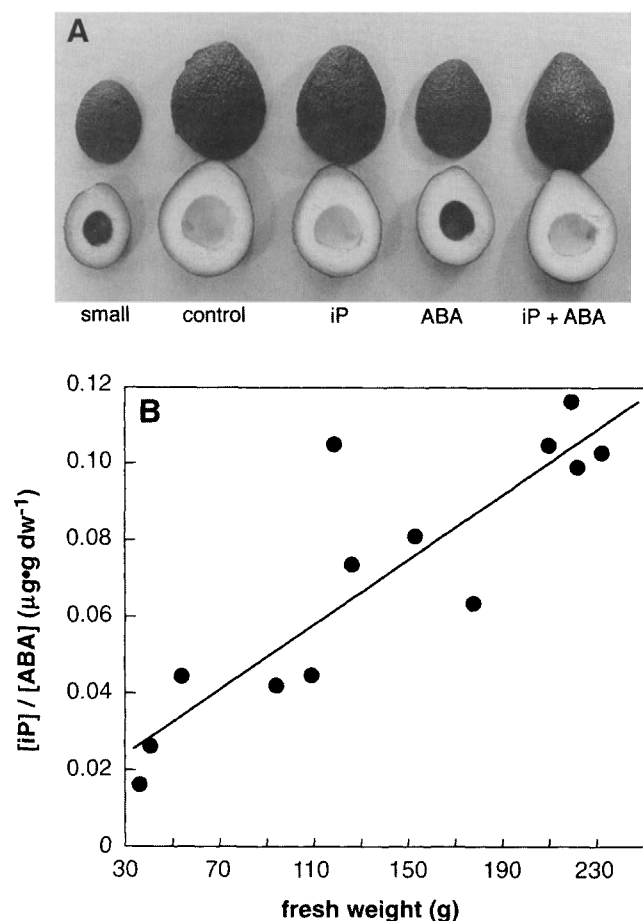


FIGURE 17-7 Relationship between cytokinin and ABA concentration and the Hass avocado small fruit variant. (A) Phenotypes of small, normal, iP-, ABA-, and ABA + iP- treated fruit. Aliquots of a solvent containing 20 μg of ABA, iP, or ABA plus iP were injected in the fruit *via* the pedicel 55 days after full bloom; fruits were harvested 226 days after full bloom. (B) Mesocarp [iP]/[ABA] content of fruit harvested 226 days after full bloom as a function of fruit size. Modified from Moore-Gordon *et al.* (1998).

common for the horticultural varieties of banana, pineapple, cucumber, tomatoes, figs, oranges, grapes, kiwi, blackberry, pepper, etc. Parthenocarpy is a genetically inherited trait, and different cultivars (or ecotypes) differ in their potential to form parthenocarpic fruit. (Mankind, of course, has selected the particular genotypes that produce fruit without seed from among the natural populations.)

In natural populations, parthenocarpy results from one of three causes: (i) lack of pollination, (ii) pollination occurs but fertilization does not, and (iii) fertilization is followed by embryo (seed) abortion. Thus, fruits of cucumber contain seed-like structures, but these structures lack an embryo and endosperm. Navel oranges and Thompson seedless grape also show seed abortion. The reason(s) for failure of

an embryo to develop or for seeds to abort is not known.

Hormones, auxins, gibberellins, and cytokinins, especially the first two, are well known to induce parthenocarpy. Thus, auxin treatment of young, unpollinated ovaries in certain cultivars of strawberry, tomato, grape, and orange is known to cause production of parthenocarpic fruit. Also, treatment of certain cultivars of oranges, tomatoes, blueberries, garden pea, and *Arabidopsis* by GA_3 (or GA_1) causes parthenocarpic fruit development (Fig. 17-8). These hormonal treatments are given to ovaries of unopened, unpollinated (or emasculated) flowers just before anthesis or during the receptive period after anthesis.

The potential to form parthenocarpic fruit varies among different cultivars (or ecotypes). One possible reason is a difference in the endogenous content of auxin and/or gibberellin in the ovaries/placenta. Cultivars with a higher potential for parthenocarpic fruit set have a higher content of hormone(s) than those with lesser or no potential. In some seedless varieties

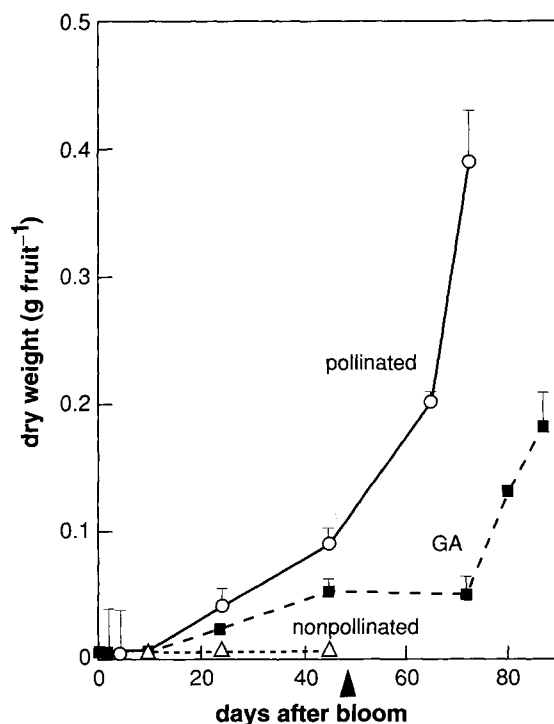


FIGURE 17-8 Changes in dry weight in GA_3 -treated but unpollinated (GA), pollinated, and nonpollinated blueberry (*Vaccinium ashei* cv Beckyblue rabbiteye) fruit. Values are means \pm SE, $n = 3$; SE bars given only when larger than symbol. Note that the final weight of GA-induced fruit was less (\sim half) than that of fruit from pollinated ovaries. Arrow indicates the time of abscission of unpollinated fruits. From Cano-Medrano and Darnell (1997).

of grape and orange, the ovaries of unopened and unpollinated flowers are reported to have a higher auxin content than those of seeded varieties. These data on auxin content were obtained from bioassays and need substantiation using GC-MS analysis. A more recent study using GC-MS correlated the gibberellin content in fruit of two cultivars of mandarin oranges, Clemantine and Satsuma. Clemantine, with a very low parthenocarpic fruit set, naturally has less endogenous GA content and responds very well to exogenous GA₃. In contrast, Satsuma, has a high parthenocarpic capability, a high endogenous GA content, and responds poorly to GA₃ application.

The exact mechanisms by which gibberellins or auxins promote parthenocarpic fruit set and development are unknown, but probably include an inhibition of presenescent changes that occur in unpollinated ovaries. The fruit that results is generally similar to that from pollinated ovaries, but some differences in kinetics of growth and fruit weight are seen (see Fig. 17-8). These differences suggest that pollination/fertilization provides other stimuli that are lacking in auxin- or GA-induced parthenocarpic fruit. It is also possible that combinations of hormones, indoleacetic acid (IAA), GAs, and CKs, are needed to duplicate the naturally pollinated fruit, but these combinations are still unknown.

4.4. Fruit Size and Shape

As mentioned earlier, the number of cell divisions in the ovary, and subsequently during early fruit growth, is an important determinant of the eventual fruit size. Hormones known to influence cell division during fruit growth include auxin and cytokinins; the role of gibberellins is less clear, although they probably are also involved. Both auxins and gibberellins are believed to be involved in cell expansion. ABA negatively regulates fruit growth, probably by inhibiting both cell division and cell growth.

It is axiomatic that the shape of the fruit would depend on the number of cell divisions and the directionality of growth of daughter cells in the various sectors of the fruit in phases II and III. For example, growth of a fruit such as tomato would require divisions in all planes and expansion of cells radially; the growth of a banana fruit, in contrast, would require cell growth mostly in a longitudinal direction; and growth of a pear fruit would require altered patterns of cell division and growth in different sectors of the fruit. Directionality of growth in fruits is probably regulated in the same manner as in growth of axial organs (see Chapter 15) by precise orientations of microtubules and innermost cellulose fibrils, but

there is little published information on this topic. Nonetheless, because GAs enhance elongation growth in stems and leaves, gibberellin sprays are used to make grape berries longer and bigger. The pedicels also elongate more, thus affecting a better separation between berries, which has the dual benefit of preventing infestation by mold and enhancing the grape's table appeal. Mixtures of a cytokinin, benzyladenine, and GA₄₊₇ have been used to shape McIntosh apples.

Because cell/organ growth involves changes in wall architecture, it is also to be expected that wall-loosening enzymes, such as endo-1,4- β -glucanases (EGases) and xyloglucan endotransglycosylases (XETs), would also be activated (for EGases and XETs, see Chapter 15 and Box 15-2). It is interesting, therefore, that an EGase gene, *Cel4* (for cellulase 4), in tomato is expressed at high levels in young growing fruits (Fig. 17-9); it is also expressed in elongating dark-grown hypocotyls of tomato. EGase genes in plants belong to large, divergent multigene families, and members of different families show tissue- and development-specific expression in growing, enlarging systems (e.g., young fruits, elongating stems) or, as explained later, during fruit ripening and during the abscission of leaves, flowers, and fruits.

4.5. Import of Photoassimilate

During their rapid growth in phases II and III, fruit act as strong sinks and import massive amounts of photoassimilate from photosynthesizing organs. Such

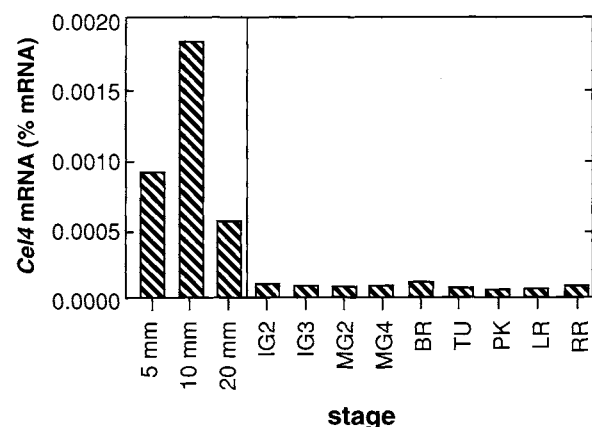


FIGURE 17-9 *Cel4* mRNA abundance in tomato fruit pericarp tissue. mRNA was extracted at different stages during fruit development and ripening: when the fruit was 5, 10, and 20 mm in diameter and at stages IG, immature green; MG, mature green; BR, breaker; TU, turning; PK, pink; LR, light red; and RR, red ripe. Data from northern hybridizations are plotted as histograms. As can be seen, *Cel4* transcripts are expressed predominantly during early fruit development. From Brummell *et al.* (1997) with kind permission from Kluwer.

translocation occurs in the phloem tissue and the translocated material is mostly sucrose, although in some species, oligosaccharides (e.g., raffinose) or hexitols (mannitol, sorbitol) may be the predominant sugar/sugar alcohol. On arrival, the sugar (sugar alcohol) may be converted to starch (e.g., mango, banana, kiwifruit), stored as reducing sugars [e.g., domesticated tomato (*L. esculentum*), strawberry (*Fragaria*)], or stored as sugar (wild species of tomato, muskmelon, water melon, grape). In some fruits, it may be converted to lipids (e.g., olive). Many of these stored foods undergo further modifications at the time of fruit ripening. Various enzymes involved in sugar-starch metabolism, e.g., acid invertases (for hydrolysis of sucrose to hexoses, fructose, and glucose), starch synthases, and starch branching enzymes, are active in growing fruits. By removing sucrose from the site of unloading, these enzymes play an important role in maintenance of the sucrose concentration gradient in the phloem stream and in the sink strength of the fruit.

Hormones have been implicated in regulating the partitioning of photoassimilate between competing sinks. For instance, auxins, gibberellins, and cytokinins all affect the sink size by promoting cell division and/or cell expansion. Whether they regulate the loading of photoassimilate in the source leaves or its unloading at the sink site in the growing fruit is uncertain. In many studies, ABA has been suggested as the hormone that facilitates unloading of the photoassimilate at the sink site, but evidence is equivocal.

5. FRUIT RIPENING INVOLVES A COMPLEX SET OF CHANGES

Phase IV, that of fruit ripening, involves a complex series of events, a change in color, softening of the pericarp (ovary wall), and changes in sweetness and flavor, that make fruits both tasty and attractive to eat. Each of these subsets involves changes in expression of many genes. Before considering these changes, it is useful to distinguish between climacteric and nonclimacteric fruits.

5.1. Climacteric vs Nonclimacteric Fruits

Ethylene is produced at a low, basal level throughout fruit development and maturation; however, in many fleshy fruits, ripening-related changes (see earlier discussion) are signaled by a sudden and dramatic rise in ethylene synthesis as well as a rise in the respiration rate (Fig. 17-10). Fruits that show such a rise in respiration rate and ethylene biosynthesis are referred to as **climacteric** fruits. In others, **nonclimacteric** fruits, the low levels of ethylene production continue during ripening and there is no rise in the respiration rate or in ethylene production.

Table 17-1 lists some of the commercially important climacteric and nonclimacteric fruits. It should be noted that climacteric-related ethylene production is seen in both harvested fruit in storage and fruit that ripens on the plant.

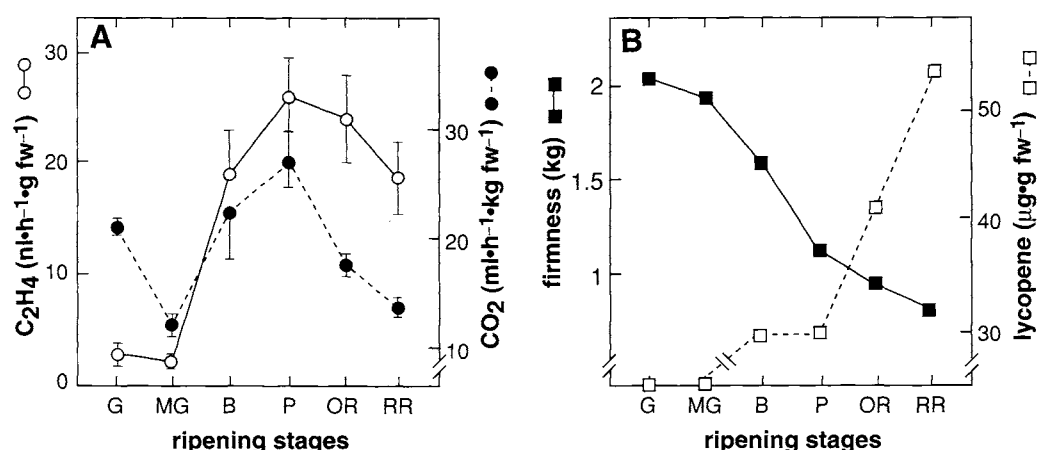


FIGURE 17-10 Relationship between climacteric, C_2H_4 production and fruit ripening in tomato (*Lycopersicon esculentum*). (A) Changes in CO_2 and ethylene production and (B) correlated decline in firmness of fruit and increase in its lycopene (carotenoid that gives the red coloration) content. Note the correspondence between ethylene production and respiration rate; also, that the rise in lycopene level coincides with the peak in ethylene production rate. Pulp firmness is the residual force after a 1-cm penetration of a 3-mm-diameter tip. G, green; MG, mature green; B, breaker; P, pink; OR, orange; RR, red ripe. Adapted from Rothan and Nicolas (1989).

TABLE 17-1 Some Common Climacteric and Nonclimacteric Fruits^a

Climacteric	Nonclimacteric
Apple	Bell pepper
Avocado	Cherry
Banana	Citrus
Cantaloupe	Grape
Mango	Pineapple
Olive	Snap bean
Papaya	Strawberry
Passion fruit	Watermelon
Peach	
Pear	
Plum	
Tomato	

^aReprinted with permission from Abeles *et al.* (1992).

5.1.1. Increase in Ethylene Production May Involve Specific Isoforms of Ethylene Biosynthesis Enzymes

In climacteric fruits, the low levels of ethylene production before the climacteric are sometimes referred to as system 1 production and the subsequent burst of high levels of production as system 2 production. As discussed in Chapter 11, ACC synthase (ACS) and ACC oxidase (ACO) are the two pacesetter enzymes in ethylene biosynthesis. Both are encoded by multi-gene families, which are induced in a tissue-specific manner by developmental, hormonal, and environmental cues. Tomato has a large family of genes encoding ACS and a smaller family of genes encoding ACO isoforms, and gene-specific probes in tomato have shown that different ACS genes are expressed under system 1 (e.g., ACS1, ACS3) or system 2 (ACS2, ACS4); in other words, different isoforms of ACC synthase are involved in low vs high levels of ethylene production.

5.1.2. Increased Respiration Rate Involves the Alternate Oxidase System

Whereas ethylene production is related to an induction of many genes (see Section 6.2), the reason for a rise in respiration rate is not clear. It has been thought that it may fuel ripening-related changes. However, the rise in respiration rate in many climacteric fruits is due to the activity of the cyanide-independent alternate oxidase system (AOX), in which oxidation of reduced carbon occurs but no energy is harnessed in the form of ATP. Thus, the usefulness of the respiratory climacteric in fueling ripening-related changes is questionable. It is also unclear what proportion of respiration is due to AOX and what proportion to the normal respiration.

Although many of the ripening-related changes in climacteric fruits, are triggered by ethylene, whereas there is no such relationship in nonclimacteric fruits, the nature of changes associated with ripening and the enzymes involved in those changes are similar between the two types of fruits. Moreover, within a species, some cultivars may show a climacteric, whereas others may not. Thus, there may be no fundamental difference between the two types of fruits. Nonetheless, in the following, for historical reasons and convenience of description, the two are considered separately.

6. RIPENING IN CLIMACTERIC FRUITS

Ripening in climacteric fruits has been well studied partly because they include many important commercial crops (see Table 17-1) and partly because, in these fruits, ethylene acts as an inducer of many genes associated with color changes, changes in carbohydrate reserves, and softening of the pulp.

6.1. Continued Ethylene Production Drives the Ripening Process in Climacteric Fruits

Until recently, it was not clear whether the ripening of climacteric fruits was induced by ethylene or whether ethylene was a by-product of the ripening process. The mystery was resolved by the use of transgenic plants. Transgenic tomatoes, where ethylene production is curtailed drastically by inserting the coding sequence of either the ACC synthase or the ACC oxidase gene in an antisense orientation, coupled to a strong promoter (e.g., CaMV S35), show no climacteric and do not ripen. Ethylene-insensitive mutants, such as *Nr* (for neverripe), in tomato do not ripen. Moreover, ethylene does not act merely as a molecular switch, which turns on the ripening process, but rather as a motor running continuously for ripening-related processes to continue. If ethylene production/action is stopped by the use of ethylene synthesis inhibitors or inhibitors of ethylene perception, the ripening process is slowed down considerably.

6.2. The Ripening Process Involves the Differential Expression of a Large Number of Genes

During ripening in tomato, many mRNAs (> 20) are up- or downregulated. Upregulated genes include

those encoding enzymes for ethylene biosynthesis, carotenoid biosynthesis, starch–sugar metabolism, wall degradation, and antioxidant action. Among those downregulated are genes for chlorophyll-binding proteins and for RUBISCO subunits. How the expression of so many genes is coordinated in a precise, orderly fashion is not known. Northern blots using ripening-related cDNA clones show that upregulated genes are expressed only during ripening. Their mRNAs are either absent or present in very small amounts in the green fruit. Thus, they are developmentally regulated. Moreover, many of them, although not all, are also induced by ethylene. Thus, there is ethylene-dependent and ethylene-independent induction.

Some genes are expressed early when ethylene concentrations are still low, whereas others are expressed later in fruit ripening as the ethylene concentration rises. Among the early expressed mRNAs are those encoding the ACC synthase and ACC oxidase enzymes. As mentioned in Chapter 11, ethylene catalyzes its own production by a positive feedback mechanism. Small amounts of ethylene induce the expression of specific ACC synthase genes, which results in the production of larger amounts of ethylene by the process of autocatalysis. These enzymes have a short half-life and are turned over at a fast rate to maintain high levels of ethylene production for a long time.

6.3. Changes in Fruit Color

The change from a green mature fruit to a ripe tomato, apple, or banana involves a transition of chloroplasts into chromoplasts. Thylakoid membranes and chlorophyll pigments are broken down, and there is a progressive accumulation of new carotenoid pigments in the plastids. In tomato, the new carotenoids include β -carotene and lycopene, which are responsible for the orange and red color of the fruit, respectively. Lycopene synthesis in tomato is induced by ethylene (see Fig. 17-10B), and is shut off by inhibitors of ethylene synthesis or perception.

As explained in Chapter 10, dimerization of geranylgeranyl diphosphate (GGDP) in a two-step process gives rise to phytoene, the first compound in carotenoid biosynthesis. The mRNA for prephytoene pyrophosphate synthase, the key enzyme in the condensation reaction of GGDP, is upregulated by ethylene. β -Carotene and lycopene arise from phytoene.

6.4. Change in Flavor

During ripening, fruits show an increase in the concentration of sugars, either by hydrolysis of starch

within the fruit or by continued import of sugars from other parts of the plant. The former is typical of fruits that store starch, which are often harvested before ripening (e.g., banana, mango, kiwifruit), and the latter of fruits that ripen on the vine (e.g., melon). Moreover, sucrose is hydrolyzed to hexoses, principally fructose and glucose. Activities of enzymes involved in starch/sugar metabolism, such as sucrose phosphate synthase and acid invertase, have been shown to rise in several fruits, and their mRNAs have been shown to be upregulated by ethylene. In addition to a change in sweetness, flavoring components, such as organic acids (principally citric acid, but also malic acid) and volatiles, increase during ripening and combine to produce the unique flavor and aroma of the ripe fruit. Phenolics, such as tannins, provide astringency to unripe fruit and have an important influence on the flavor and color of mature fruit.

6.5. Softening of the Pericarp

Various hydrolases are required for the degradation of cell walls and softening of the pulp. They include enzymes that disrupt the hemicellulose–cellulose network, as well as those that disrupt the pectin network (see Table 17-2). Genes for these enzymes have been cloned and described from many fruits, e.g., tomato, avocado, peach, banana, and passion fruit.

As mentioned earlier, EGases are a large group of enzymes expressed in a tissue- and development-specific manner (see Section 4.4). EGases expressed during fruit ripening are similar in some ways to those induced during organ abscission; both are induced by ethylene and are downregulated by auxin (Fig. 17-11). In contrast, EGases expressed during fruit growth in phases II and III are similar to those expressed during stem elongation and are induced by auxin (see Fig. 17-9). These two classes of plant EGases may also differ in their *pI* values: those expressed in ripening fruit and during abscission are reported to have a *pI* of 9.5, whereas those expressed during stem expansion and early fruit growth have a *pI* of 6.5.

TABLE 17-2 Enzymes That Disrupt Cell Wall Architecture in Ripening Fruits

Hemicellulose–cellulose network	Pectin network
Expansins	Polygalacturonases
Xyloglucan endotransglycosylases	Pectin methylesterases
Endo-1,4- β -glucanases (EGases or cellulases)	
α - and β -galactosidases	

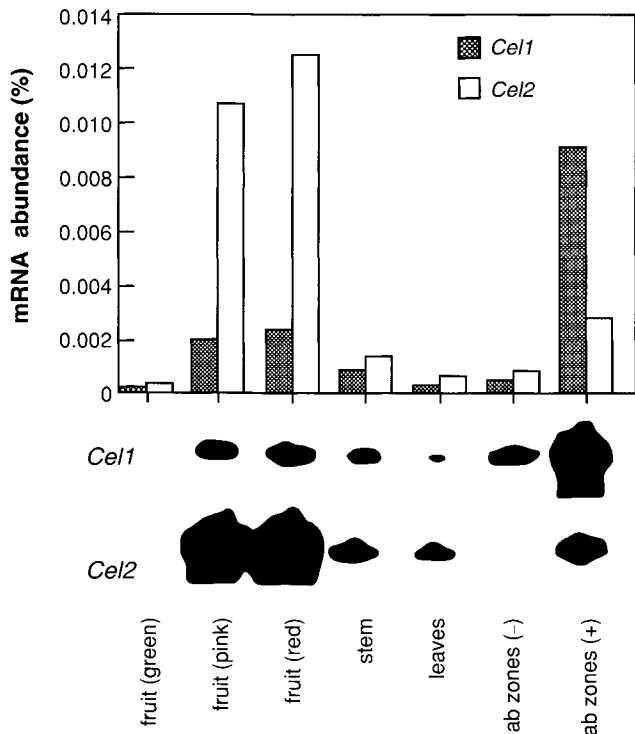


FIGURE 17-11 Expression of two EGase genes, *Cel1* and *Cel2*, in tomato. Both are induced by ethylene and inhibited by auxin, but *Cel1* is expressed primarily in the abscission zone, whereas *Cel2* is expressed primarily in ripening fruit. Fruit RNA was isolated at three stages of ripening. RNA from stems and leaves were isolated from field-grown plants. Abscission zones were isolated from floral explants that abscised (+ ab) or failed to abscise (- ab) after being held in water-filled tubes. Histograms show mRNA abundance as the percentage of poly(A)⁺ mRNA present in each tissue. Northern blots for *Cel1* and *Cel2* RNA appear below the histograms. From Lashbrook *et al.* (1994).

Genes encoding expansins and XETs also belong to multigene families, and members expressed during fruit ripening in tomato are ethylene induced.

One of the first genes to be cloned for fruit ripening, and among the best studied, is the *PG* gene in tomato that encodes the enzyme polygalacturonase (PG). PG catalyzes the hydrolytic cleavage of α -(1-4)galacturonan linkages and, thus, is the key enzyme for affecting large changes in pectin structure. In tomato, there is a large family of *PG* genes. In ripening fruit, however, only one *PG* gene has been identified, which produces three isoenzymes, PG1, PG2a, and PG2b, by posttranslational modifications. Why three isoforms of PGase are needed is not clear, although it has been suggested that PG2 isoforms are involved in the degradation of polygalacturonic acid (PGA) polymers into oligomeric fragments of smaller molecular weight, whereas the PG1 isoform is involved in the breakup of oligomers into galacturonic acid residues.

The production of PG is under developmental control: the enzyme is absent in green, mature fruits and is synthesized *de novo* during ripening. Its production is also regulated by ethylene at both transcriptional and translational levels. Mature green fruits, but not young developing fruits, treated with ethylene show an accumulation of *PG* mRNA. The levels decline if ethylene is withdrawn or if the ethylene perception pathway is blocked by silver thiosulfate or 2,5-norbornadiene. This decline occurs even after ripening has started, which confirms that ethylene acts as a continuous regulator of ripening, not just as an on/off switch. That the expression of the *PG* gene is also under translational control is shown by tomato fruits that have been transformed by an antisense insertion of an ACC synthase gene. In these fruits, endogenous ethylene production is reduced by more than 99% of that in untransformed plants; hence, the effect of exogenous ethylene can be monitored easily. These fruits treated with exogenous ethylene at the mature green stage show an accumulation of *PG* mRNA in a concentration-dependent, time-specific manner, but do not show much *PG* polypeptide or *PG* activity (Fig. 17-12). These fruits, with low endogenous ethylene, accumulate sufficient amounts of *PG* protein over time (4 to 6 days) or, alternatively, if supplied with ethylene (or its analog propylene) in large quantities. The tomato mutant *Nr*, which is insensitive to ethylene (see Chapter 21), produces *PG* mRNA but not the protein product unless ethylene is present, which also suggests translational control.

It would be expected that the enzyme PG is responsible for softening of the fruit pulp, but such is not the case. If PG production is curtailed in tomato plants by transforming them with constructs containing the coding sequence of the *PG* gene in an antisense orientation, there is no significant change in the softening of the transgenic fruit relative to that in the control, even though pectin degradation is inhibited. This result clearly indicates that other factors, primarily the breakdown of the cellulose-hemicellulose network, are responsible for the loss of firmness in the ripening fruit.

In the wall, a substantial part of the polygalacturonic acid (PGA) exists in an insoluble methylated form (see Chapter 2). Pectin methylesterase (PME) removes the methyl groups from PGA, thus making it available for action by PGs. Two isoforms of PMEs are known from tomato, and the activity of one increases several-fold during ripening.

6.6. Other Genes

Among other genes, a gene for superoxide dismutase (*SOD*) and another for a catalase enzyme have

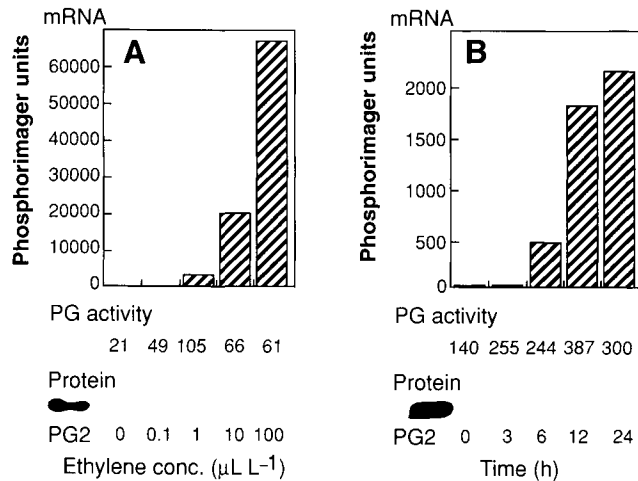


FIGURE 17-12 Accumulation of PG mRNA and PG protein in transgenic tomato fruit treated with exogenous ethylene. Transgenic tomato fruits were prepared by antisense expression of an ACC synthase gene. (A) Effect of ethylene concentration on PG mRNA and PG protein accumulation. Transgenic fruit were treated with different concentrations of ethylene for 24 h. As can be seen PG mRNA increased in a concentration-dependent manner between 0.1 μM and 100 μM ethylene, but there was little increase in PG protein or activity. (B) Kinetics of PG mRNA and PG protein accumulation at a defined ethylene concentration (10 μM · liter⁻¹). mRNA accumulation started 6 h after ethylene treatment and reached maximal levels between 12 and 24 h, but there was little increase in PG protein or activity. In both figures, PG mRNA accumulation was based on radioactivity in the Northern hybridization blot estimated with a phosphorimager and plotted as histograms. PG enzyme assays were performed by incubating 10 mg of crude cell wall protein extracts with the substrate, polygalacturonic acid. PG protein was detected with an immunoluminescent antibody. PG2 indicates the mobility of purified PG. From Sitrit and Bennett (1998).

been identified in ripening tomato. Both enzymes have known antioxidant activities and are induced by stress or pathogenesis, but their role in fruit ripening is not clear.

6.7. Subsets of the Ripening Program Are Independently Controlled

Tomato mutants are available where one or another subset of changes may be impaired. Thus, in mutants, such as *yellow flesh*, *greenflesh*, and *tangerine*, only carotenoid biosynthesis or chlorophyll degradation is affected, not the softening of the flesh or starch-sugar conversions. In mutants such as *ripening inhibitor* (*rin*) and *nonripening* (*nor*), other aspects of ripening, such as pulp softening and flavor, are also affected. The existence of these mutants indicates that subsets of the ripening program, while under the overall regulation by ethylene, may be controlled independently.

In an excellent demonstration of this phenomenon, as well as the fact that softening of the pulp is not due to the activity of the PG enzyme, the *rin* mutant was transformed with a construct consisting of the PG-coding sequence under the control of a promoter that could be induced by exogenous ethylene. On supply of ethylene to the transformed mutant plants, PG mRNA was produced, pectins were hydrolyzed, and the fruit changed color, but it failed to become soft.

7. RIPENING IN NONCLIMACTERIC FRUITS

Ripening of nonclimacteric fruits has not been studied to the same extent as in climacteric fruits, although information has begun to accumulate for some fruits such as strawberry, citrus, and grape.

The ripening of strawberry involves many of the same changes that are seen in the ripening of climacteric fruits, i.e., loss of chlorophyll and acquisition, in this case, of anthocyanins; accumulation of sucrose, hexoses, and flavoring volatiles, such as alcohols, aldehydes, and their esters; and softening of pulp. Among these, accumulation of anthocyanins is an easily visible marker, and has been used to judge ripening (Fig. 17-13). Many studies have shown that (1) ripening in strawberry fruit is not affected by exogenous ethylene and (2) ethylene biosynthesis inhibitors (e.g., aminoethoxyvinylglycine, AVG) or inhibitors of ethylene action (e.g., silver thiosulfate or 2,5-norbornadiene) do not affect strawberry ripening.

Since developing achenes are a rich source of auxin, and exogenous application of auxin to

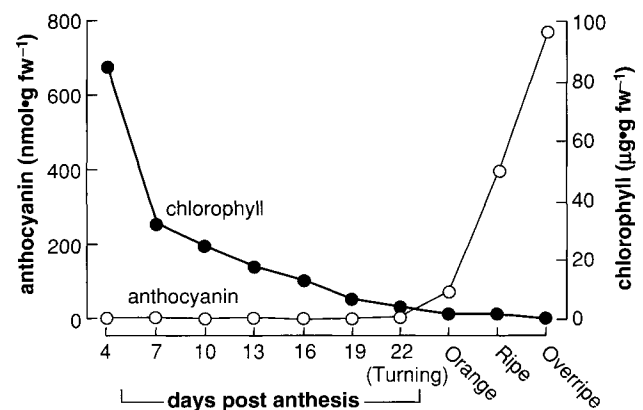


FIGURE 17-13 Changes in chlorophyll (●) and anthocyanin (○) content of strawberry fruit during development. Receptacles were sampled at different stages of development and pooled for extraction of chlorophyll and anthocyanins. From Manning (1994).

deachened segments of strawberry fruit stimulates the growth of young fruit, the question arises whether auxin has a role in ripening. Deachened fruits accumulate much more anthocyanins than the control, and application of auxin to deachened segments results in a much reduced accumulation of anthocyanins (Fig. 17-14). These data indicate that auxins retard or inhibit ripening-related changes in strawberry. They also mean that, as the achenes mature, free IAA must be inactivated or broken down in order for the strawberry fruit to ripen.

Several ripening-related cDNA clones in strawberry have been isolated and sequenced. Many encode enzymes in anthocyanin biosynthesis (phenylpropanoid pathway). mRNAs of these enzymes are down-regulated by the treatment of green, mature fruits with auxin. Among the sequenced clones, none has been

reported so far to encode ACC synthase or ACC oxidase enzymes, which, as mentioned earlier, are upregulated during the ripening of climacteric fruits.

Softening of the pulp in strawberry fruit is accomplished by an array of endo-1,4- β -glucanases and pectinases similar to those in climacteric fruits, but their induction is not linked to ethylene. An expansin, homologous to tomato fruit expansin, has also been identified. An unusual pectinase gene, that for pectate lyase, which is known mostly from fungal pathogens, has been cloned from strawberry and banana fruit and is expressed during ripening.

Alcohol acyltransferases (AATs) are enzymes responsible for the production of esters that contribute to the distinctive tastes and aroma of many fruits. A gene encoding an AAT from strawberry has been isolated using the technique of DNA microarrays (see Appendix 1). The gene, called *SAAT*, is expressed much more highly in ripe red fruit than in green or white fruit. Its functional expression in *Escherichia coli* confirmed that it encodes an AAT.

Grape berry and citrus fruit are two other nonclimacteric fruits. In grape, as in strawberry, auxin treatment retards ripening. Citrus fruit is unusual; although it is a nonclimacteric fruit, carotenoid synthesis in the orange peel is ethylene regulated.

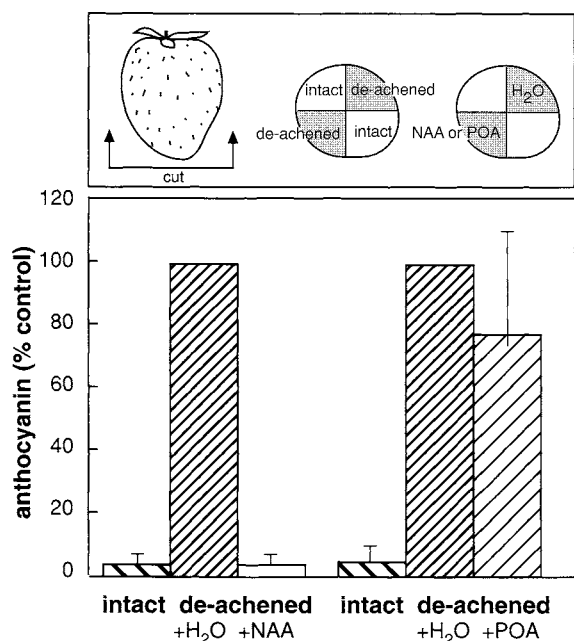


FIGURE 17-14 Effect of 1-naphthalene acetic acid (NAA, a synthetic auxin) and phenoxyacetic acid (POA, an inactive auxin analog) on the anthocyanin content of deachened strawberries. Achenes were removed from opposite quarter segments of mature green fruit, and 0.5 μ l of a solution of NAA (2 mM) was applied with a microsyringe to each of the achene recesses on one deachened segment of each fruit (see schematic on top). Water was added to the opposite deachened segment (control). Another group of fruit was treated similarly except that POA replaced NAA on the treated side. Fruit were sampled when the control segments had ripened, but before accumulation of anthocyanin occurred in the intact segments. Anthocyanin content per gram fresh weight was measured in each deachened segment and in the intact receptacle segments and was calculated as a percentage of the control value for each fruit. $n =$ six for each treatment. Values are means \pm SD. From Manning (1994).

8. POSTHARVEST CHANGES AND FRUIT STORAGE

Many climacteric fruits, such as banana, tomato, peach, and pear, are harvested when fully mature, and ripening is controlled while the harvested fruit is under storage. Control is exercised mainly by regulating the levels of ethylene. Excessive ethylene production by ripening fruit results in early deterioration of fruit quality and poor shelf life. Ethylene levels can be regulated to some extent by the use of inhibitors of ethylene synthesis (AVG, AOA) and/or ethylene action (e.g., silver thiosulfate, 2,5-norbornadiene, *trans*-cyclooctene). However, use of these compounds in the food industry is not acceptable; moreover, silver is a heavy metal, and norbornadiene and *trans*-cyclooctene have a foul odor and need to be used in large concentrations. The search for more acceptable alternatives has led to the use of cyclopropene compounds, especially 1-methylcyclopropene (1-MCP), which is an odorless gas effective in small doses and whose effect persists for 2–3 weeks at cool temperatures). Transgenic approaches in which ethylene production by the fruit is curtailed are also being tried (see Section 10).

Environmental factors, such as temperature, and CO₂ concentration affect the quality and ripening of climacteric fruits. Species vary in their tolerance levels to high temperature and elevated CO₂ levels. Enhanced temperature, above 30°C, generally inhibits ripening-related changes, probably by inhibiting ethylene production. The activities of various enzymes, including ACC synthase, ACC oxidase, PG, and acid invertase, are known to be affected by high temperature. Likewise, CO₂ concentrations above 4–5% affect ripening adversely by inhibiting ethylene biosynthesis.

9. FRUIT ABSCISSION

Abscission of mature fruit, as that of leaves and flower pedicels, is regulated by ethylene and is inhibited by hormones such as auxins, gibberellins, and cytokinins. Abscission of plant organs is covered in Chapter 20.

10. PRODUCTION OF TRANSGENIC FRUIT

A knowledge of the enzymes associated with fruit ripening allows a manipulation of those enzymes to regulate the rate of ripening and also to introduce desirable qualities of sweetness, flavor, and color in genetically engineered fruit.

Because ethylene is a major inducer of genes associated with ripening in climacteric fruits, control over ethylene production can be used to regulate the rate of ripening. Such control has been exercised by introducing the ACS or ACO genes in an antisense orientation or by reducing the supply of ACC, the immediate precursor of ethylene, by deaminating it (see Fig. 11-13 in Chapter 11). Such deamination was accomplished by introducing a bacterial gene for ACC deaminase. Production of transgenic tomato fruit by these two methods was accomplished several years ago, and a transgenic tomato with an enhanced shelf life has been available commercially for many years. In more recent years, similar techniques have been used to enhance the shelf life of other fruits, e.g., melon.

The color of a fruit can be modified by enhancing or silencing the expression of specific genes encoding key enzymes in carotenoid or anthocyanin biosyntheses. Similarly, the sweetness of fruit, or a specific flavor, may be modulated by manipulating the expression of genes for sugar metabolism or genes involved in the synthesis of a specific flavoring compound.

A novel approach is to use common and relatively inexpensive fruit, such as banana, to deliver oral vaccines or other drugs to human or animal populations. Some mRNAs are produced in great abundance in certain fruits, e.g., PG mRNA in tomato, which may account for as much as 1–2% of total RNA at peak times. If PG (or some other) mRNA is produced in abundance in banana, it is conceivable to link the gene for a key enzyme in the production of a drug to the promoter sequence of the PG gene for its expression in the ripening banana.

Production and marketing of transgenic fruit for consumption by humans (or cattle) inevitably raise ethical and moral questions, as well as questions about safety and health. A consideration of those topics is beyond the scope of this book.

11. CHAPTER SUMMARY

Fruit growth is a complex phenomena, which is divided into four phases. Phases I–III involve growth of the fruit from a mature, unpollinated ovary to the fully developed but unripe fruit; phase IV involves ripening of the fruit. The growth of the fruit to maturity and ripening are regulated by distinct hormonal and environmental signals.

Pollination and fertilization provide strong stimuli for retention of the young fruit on the plant and for early fruit growth. Three hormones, auxins, gibberellins, and cytokinins, are involved in the retention of young fruit on the plant and in the promotion of fruit growth by enhanced cell divisions and cell enlargement. Auxin (IAA) and cytokinin concentrations are high in fruits at a very early stage, which coincides with the period of maximal cell division. An exogenous application of auxin or gibberellin promotes fruit set and parthenocarpic fruit development in many plants if applied to young ovaries within the receptive period. Moreover, the endogenous concentrations of IAA or GAs correlate with the capacity of a cultivar to produce parthenocarpic fruit. The differential ratios of GAs and CKs may underlie the differential growth in different sectors and shaping of the fruit. Growth of fruits to mature size involves import of photoassimilate from other parts of the plant. These hormones, by inducing cell division and cell growth, enhance the sink strength of and thus facilitate the translocation of photoassimilate to the growing fruit, but whether they affect the photoassimilate partitioning or its loading at the source leaves or unloading at the sink site is unclear. Enzymes involved in starch–sucrose metabolism are active in growing fruits. By converting

sucrose to hexoses (for storage or for starch synthesis), these enzymes help maintain a sucrose concentration gradient between the source leaves and the young fruit and thus determine the rate of assimilate transport to the fruit.

Fruits undergo profound physiological and biochemical changes during ripening, which include a change in color, changes in taste and flavor, and changes in firmness. Fruits are classified into climacteric and nonclimacteric fruits. While both types show low basal levels of ethylene production throughout fruit development, in climacteric fruits, ripening-related changes are signaled by a sudden and dramatic increase in ethylene biosynthesis and in respiration rate. The reason for the increase in respiration rate is not clearly understood, but the increase in ethylene production serves as the motive force for the induction of many genes, including genes for its own synthesis (autocatalysis), as well as genes for carotenoid biosynthesis, for various wall hydrolases, and for enzymes in starch-sugar metabolism. How such multiple sets of genes are regulated is not known. The ripening-related changes in nonclimacteric fruits are similar, but there is no correlation between ethylene levels and induction of various enzymes. While ethylene does not promote ripening in these fruits, at least in some cases, treatment with auxin has been shown to inhibit ripening.

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Seed Development and Maturation

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1. A SEED IS A MARVELOUS PACKAGE

A seed is a marvelous adaptation for survival of the embryo for long periods of time, often under adverse environmental conditions. Such survival allows opportunities for dispersal, both in time and in space. During this period, attack by bacteria and fungi is discouraged by the desiccated state of the seed as well as by the hardened seed coat¹; also, in many seeds, the presence of phenolics, lectins, toxic glycosides, and enzyme inhibitors discourages predation by insects, rodents, and herbivores. A seed usually comes with everything it needs for germination and early seedling growth, including reserve food and minerals, and at that time requires only the right temperature, H₂O, and O₂ from the environment. (Seeds of a few species are known to germinate under anoxia, but their numbers are very small.) Moreover, in many cases, the seed, more properly the embryo, exercises its own control on germination—it determines when the environmental conditions are right for germination.

2. STAGES IN SEED DEVELOPMENT

As in fruit development, seed development also occurs in defined although continuous steps. Most of

¹Seed coat is a nontechnical term for testa or the modified integument(s); in some cases, it also includes the fruit wall (pericarp) and surrounding tissues.

the information so far comes from flowering plants; hence, the emphasis in this chapter is on them, although some important differences with conifers are indicated in appropriate places.

Seed development is often described on the basis of stages of embryo development, i.e., early, mid-, and late embryogenesis. These stages are not sharply defined, they partly overlap, and should be visualized as part of a continuum with shifting major activities (Fig. 18-1). In some texts, these stages are referred to as histodifferentiation (phase I), expansion (reserve deposition) (phase II), and maturation drying (phase III).

2.1. Early Embryogenesis

Early embryogenesis is characterized by the laying down of the body plan of the embryo, i.e., the definition of the cotyledons (a single cotyledon in monocots), the root-shoot axis, the root and shoot apices, and the demarcation of the primary tissues: proto-

derm, ground meristem, and provascular tissues or procambium (see Chapter 1). It is also characterized by nuclear divisions and wall formation, in what was the central cell of the embryo sac, leading to cellularization of the endosperm. In conifers, embryo development follows a slightly different course, but the laying down of the body parts and the patterning of tissue layers probably occur in a similar manner. In conifers, there is no endosperm; instead the embryo develops within the female gametophyte, which is a maternal tissue.

2.2. Midembryogenesis

After the body pattern of the embryo has been set and differentiation of primary tissues has occurred, cell divisions decrease in frequency and eventually stop, but the embryo continues to grow by cell enlargement until it reaches its full size and then growth is arrested and the seed enters a period of rest or

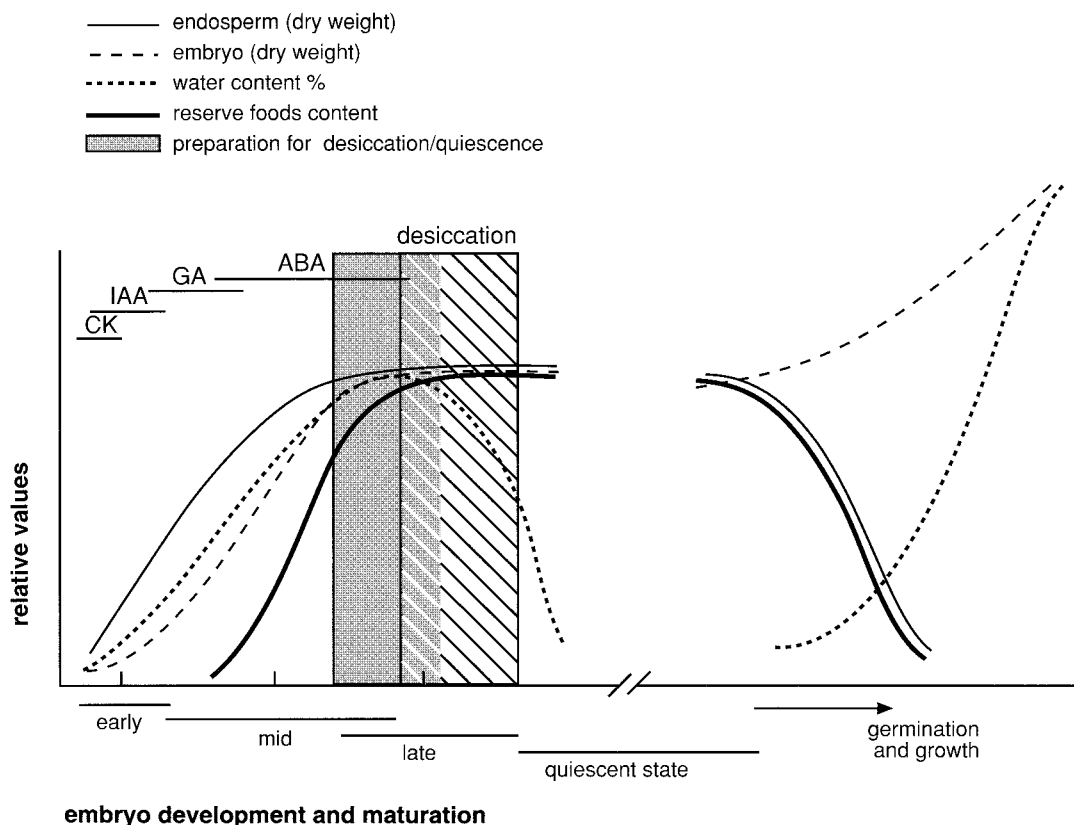


FIGURE 18-1 A generalized scheme illustrating embryo (and seed) development and maturation in flowering plants. Relative values indicate roughly when the maximum water content, embryo growth, and so on are reached. Approximate times when amounts of free hormones are high are indicated by bars. Deposition of food reserves begins with the expansion phase of the embryo and is completed about the time desiccation begins. The approximate time for synthesis of desiccation- and stress-related proteins is indicated by shading. The scheme shows an endospermic seed, where the endosperm persists and is the chief food storage tissue.

quiescence. Growth does not resume until after seed germination. In many dicots, such as lettuce, pea, bean, and sunflower, reserves in the endosperm are resorbed by the developing embryo, and at seed maturity that tissue is represented by a thin papery layer a few cells in thickness. In many other plants, including castor bean, all cereal crops, and coconut, the endosperm continues to grow by cell division and enlargement and eventually becomes much larger than the embryo. The endosperm consists mainly of living cells, but in some families, it consists of both living and dead cells at maturity. In cereals (Gramineae) for instance, the outer cell layer(s) becomes distinguished as the aleurone tissue (or layer), distinct from the central cell mass, which becomes the starchy endosperm. At maturity, while cells in the aleurone tissue continue to live, those in the starchy endosperm are dead. Seeds in which endosperm persists are referred to as **albuminous** (or **endospermic**) seeds vs **exalbuminous** (or **nonendospermic**) seeds where it does not.

This period is also characterized by an accumulation of food reserves. The types of seed food reserves and their accumulation are topics of extreme importance for human and animal food supply. A brief overview of the nature of stored reserves and the manner in which they are accumulated is given in Appendix 3—Seed food reserves and their accumulation, at the end of Section III of this book.

2.3. Late Embryogenesis

During the late stages of embryogenesis, the seeds lose water, desiccate, and “mature”.² The seed coat hardens (in some seeds, the walls of epidermal cells become lignified), and the outer walls get coated with cutin, which in some cases may be impregnated with waxy deposits. Cells may also accumulate phenolics and, in some cases, toxic materials against pathogens and herbivores.

Near the end of reserve food accumulation (seed filling), vascular connections *via* the funiculus between the mother plant and the seed become nonfunctional or are broken. As a result, the maturing seeds lose water and desiccate while still on the mother plant, although desiccation may continue after the seeds are shed. The dry weight stays constant, but the fresh weight drops. The moisture content of mature seeds,

²There is no good definition of seed maturation—some authors consider the end of seed filling as “physiological” maturation, but many important events continue or occur beyond seed filling. In this book, maturation is used loosely to designate the sum total of morphological and biochemical changes that occur from peak accumulation of food reserves to the time of seed shed.

at the time of seed shed, in many species is reduced to 10–15%, although lower values are known. In this dry state, most of the metabolic processes, including respiration, are reduced to very low or undetectable levels—a state of suspended animation known as **quiescence**.

This dry state not only allows seeds longer viability under extreme environmental conditions, but also protection against attack by bacteria and fungi. The process of desiccation, however, creates many stresses, the principal ones being increased salt concentration in the cell, crystallization of molecules, creation of toxic oxygen radicals, and attendant danger of damage to cell macromolecules and membranes. Plants have evolved several mechanisms to deal with dehydration (see Chapter 16), and some of these are utilized in seed development (see Section 4.2).

Seeds that desiccate and become quiescent are known as “orthodox” seeds. In contrast, seeds of many species do not mature in the above sense; they remain hydrated at the time of seed shed (moisture content 50–60%) and are unable to withstand desiccation. These seeds are referred to as “unorthodox” or “recalcitrant” seeds because they are intolerant of desiccation and are damaged easily or killed on drying. They are discussed in Section 7.

2.4. Seed Quiescence and Seed Dormancy

Seeds of orthodox species mature and become quiescent, but will germinate given water, oxygen, and suitable temperature conditions. In some orthodox species, in addition to becoming quiescent, seeds also become dormant, i.e., they do not germinate under favorable conditions of water, temperature, and air. Dormant seeds require some other signal, usually an environmental cue such as temperature or light, before they will germinate. Thus, there is a clear distinction between seed quiescence and seed dormancy. Since dormancy is induced during seed development, its induction is covered here, but because it becomes operational only in relation to seed germination, it is covered mostly in Chapter 19.

3. CYTOKININS, INDOLEACETIC ACID, AND POSSIBLY GIBBERELLINS REGULATE EARLY STEPS IN SEED DEVELOPMENT

Many reports based on the analysis of whole seed extracts suggest that high amounts of auxin-, cytokinin

(CK)-, and gibberellin (GA)-like substances are present during early embryo growth and seed development.

Young immature seeds and kernels have traditionally been used as important sources of cytokinins and gibberellins. Cytokinins are common in the liquid endosperm of maize and coconut; in fact, zeatin, a natural cytokinin, was first isolated from maize endosperm. GAs are common in immature seeds of pea, bean, and wild cucumber, and indeed the synthetic pathway for GAs was elucidated using cell-free extracts from these seeds. However, precise and accurate determinations of hormone concentrations in young embryos, embryonic parts, or endosperm using gas chromatography-mass spectroscopy or immunoassays are still very few. Part of the problem, of course, is the difficulty of securing enough isolated embryos or endosperm at a young stage for analysis, but improvements in the techniques of immunolocalization of hormones *in situ* and the requirement of only a few milligrams of tissue for the determination of hormone content by GC-MS (see Chapter 5) are likely to add substantially to our knowledge in this area.

Available data suggest that the concentrations of cytokinins, such as zeatin, zeatin riboside, and indoleacetic acid (IAA), are high in early embryogenesis, when cell divisions in the endosperm and embryo are abundant, and cell growth, as well as pattern formation, occurs (Fig. 18-2). This is not surprising because there is increasing evidence that these two hormones play key roles in modulating the cell cycle (see Chapter 15). In isogenic lines of barley that differ in grain size, the larger size line had higher CK content at the early embryo stage than the line of smaller size. As discussed in Chapter 14, IAA is also involved in regulating pattern formation and bilateral symmetry in embryos.

Although young seeds of several species accumulate high concentrations of gibberellins, there are very few studies that have correlated changes in GA content with seed development. In one study on seeds of Dwarf pea cv progress #9, GA₂₀ content increased rapidly between days 18 and 22 after anthesis and coincided with the period of maximal growth of the embryo, as reflected by the fresh weight of seeds (Fig. 18-3). Small amounts of GA₉ and GA₁₇ also reached peak levels slightly earlier or coincident with the GA₂₀ peak (not shown). The amounts of GA₂₀ declined subsequently whereas those of the biologically inactive GA₂₉ and GA₂₉ catabolite increased. These data can be interpreted to mean that GA₂₀, although itself inactive (see Chapter 7), could have given rise to the biologically active GA₁ during (and possibly before) the period of maximal growth and later, when embryo growth was nearly complete, was catabolized to GA₂₉

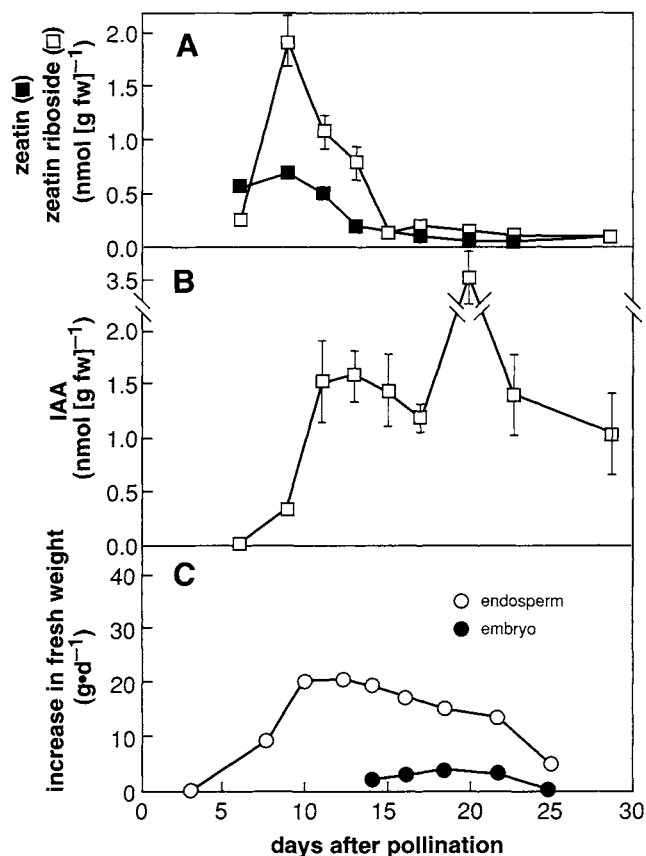


FIGURE 18-2 Cytokinin and IAA contents of developing kernels of maize (*Zea mays*). Changes in concentrations of zeatin and zeatin riboside (A) and of IAA (B) in maize endosperms. Means \pm SE for five replicates are shown. (C) Rate of fresh weight increase in endosperm and embryos. Means of five replicates are shown. Modified from Lur and Setter (1993).

and the GA₂₉ catabolite. It will be recalled, however, that dwarf pea is deficient in 3 β -hydroxylase activity that converts GA₂₀ to GA₁ in shoot tissues of young seedlings. Whether this block occurs during seed development is unknown.

Evidence from other GA-deficient mutants is equivocal. Seeds in GA-deficient mutants of *Arabidopsis* (*ga1*) and tomato (*gib1*) develop normally, although it is possible that these mutants are leaky. Notably, the GA-deficient mutant of pea, *lh-2*, in a homozygous state, produces much smaller seeds than the wild type and almost 50% seeds abort in the pods. The effects are less pronounced in the allelic mutant *lh-1*. For the *lh* mutant, it has been suggested that the smaller size of seeds and enhanced abortion are not due to limitations on the availability of photoassimilate, rather GAs are required for some process(es) essential for normal embryo and seed development. In summary, GAs probably have a role in the growth of embryo or embryo and endosperm, but more

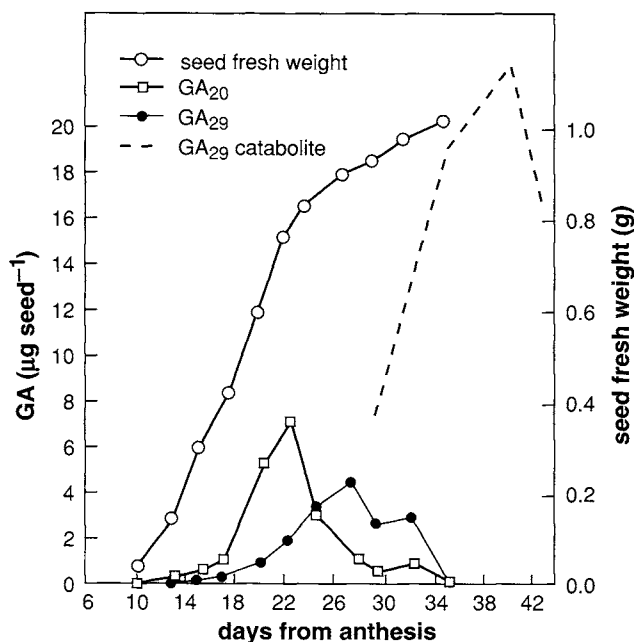


FIGURE 18-3 Gibberellin content in developing seeds of dwarf pea (cv Progress #9). The rise in GA₂₀ accompanies the increase in seed fresh weight (at this stage, pea seeds have an embryo and seed coat; the endosperm is mostly resorbed). Inactive GAs, GA₂₉, and GA₂₉ catabolite increase in amounts in later phases of seed maturation. Modified from Frydman *et al.* (1974).

studies on the distribution in seed tissues of GAs recognized to be biologically active (e.g., GA₁ or GA₄), are needed.

It is possible that these three hormones appear in a sequence, first cytokinins and IAA, when cell divisions are high and pattern formation occurs; later GA when embryo and endosperm are enlarging (see Fig. 18-1), but more work is needed before a firm conclusion can be made. The source of these hormones in early embryogenesis, whether they are derived from the mother plant or synthesized *in situ*, is also unclear. The suspensor, which is derived from the zygote, has been implicated in the supply of both CKs and GAs to the embryo. Other data, still very few and mainly on IAA, suggest that the globular and later stage embryos synthesize their own hormones *in situ* (see also Section 2 in Chapter 14).

As embryogenesis proceeds, the concentration of each of these hormones in a free form declines. They become conjugated with sugars or sugar alcohols or, in the case of IAA, with amino acids as well and are rendered inactive. In mature seeds, these hormones are present mainly as inactive, conjugated esters, or amides, and the amounts of free, active forms are relatively low. These conjugations are reversible, and it is thought that at germination some of the free hormone supply comes from the hydrolysis of conjugates.

4. ABSCISIC ACID (ABA) PLAYS A CENTRAL ROLE IN SEED MATURATION AND DORMANCY

The ABA concentration is low or negligible in early stages, but rises in midembryogenesis, when the contents of other hormones are declining, and reaches a peak about midway from seed initiation to maturity (Fig. 18-4A). This rise in ABA content is related to several important processes: prevention of precocious germination, synthesis of proteins related to protection against desiccation or damage by free radicals or other stresses, reserve food accumulation, and, in general, completion of the developmental program. In addition, ABA induces seed dormancy, if dormancy occurs. The full range or identity of the functions of ABA or of the proteins synthesized at this time is still far from clear and offers a substantial challenge to future research. After these events are initiated or completed, the free ABA content declines to almost undetectable amounts, although conjugated forms of ABA continue to be present in mature, quiescent seeds. Some ABA in seeds, especially in tissues such as the placenta/chalaza and integuments, is derived from the mother plant *via* the funiculus, but that in the embryo and endosperm is probably synthesized *in situ*.

4.1. ABA Prevents Precocious Germination

Embryos in developing seeds, after a certain stage, are capable of germinating if excised from the seed and placed on minimal culture media with minerals and a carbon source (see Fig. 18-4B). The question arises: What keeps them from germinating while the seed is still developing? One approach is to check the effects of added ABA on excised embryos. If excised embryos are placed on a medium to which ABA (1–100 µM) has been added, they mature and become quiescent; they may also synthesize LEA proteins (see Section 4.2), as well as storage food materials utilizing nutrients in the culture medium. If ABA is not added or if ABA is washed off soon after its addition by soaking the embryos in water (i.e., before ABA-induced changes have been initiated), they continue the germination program and develop into a seedling (Fig. 18-5). These studies have been performed with isolated embryos from a number of species, including wheat, cotton, rapeseed, and french bean. While there are variations as to whether the embryo develops normally or abnormally, or whether food materials are accumulated or not, there is little doubt that ABA

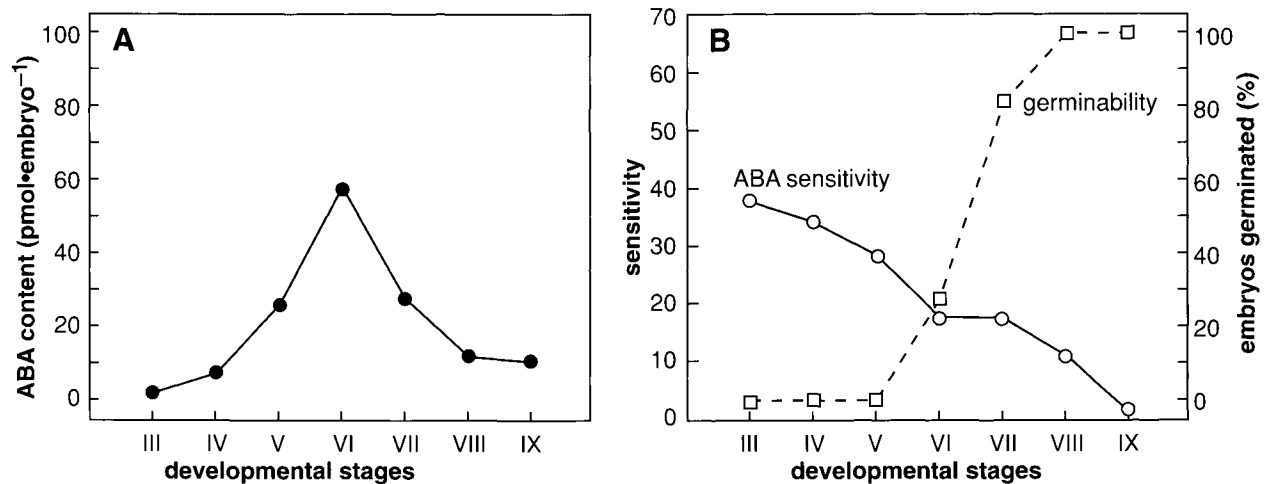


FIGURE 18-4 ABA, seed development, and germination of excised embryos in alfalfa (*Medicago sativa*). Stages III to VI correspond to midembryogenesis and VI through IX to late embryogenesis. (A) The free ABA content increases during midembryogenesis, rises to a peak at about the end of that period, and then declines during late embryogenesis. From Xu *et al.* (1990). (B) If the embryos are excised, they germinate readily after a certain stage, and the percentage inhibition by exogenous ABA, a measure of their sensitivity to ABA, declines as they mature. Reprinted with permission from Xu and Bewley (1991), © Oxford University Press.

promotes maturation and desiccation tolerance in embryos.

Somatic embryos normally have no maturation/quiescence phase and proceed right on to germinate (see Chapter 3). However, if they are incubated with

ABA, they can also be induced to synthesize reserve foods, mature, and become quiescent.

Studies with embryos in culture have also utilized solutions of high osmolarity as a replacement for ABA. In many of these studies, the induction of maturation and desiccation tolerance, similar to that induced by ABA, has been noted. High osmolarity mimics some, although not all, changes brought on by ABA. For instance, many stress-related genes are induced by both ABA and high osmoticum, although they involve different signaling pathways (see Chapters 16 and 23).

4.1.1. Viviparous Plants

Support for the role of ABA in the prevention of precocious germination comes from plants that produce viviparous seeds. Mangroves are marine plants that grow in swampy, estuarine, or intertidal waters in the tropics. Some mangrove species produce seeds that can be considered “mature”; however, unlike those of most plants, they do not dry or become quiescent. Instead, they germinate precociously while still on the mother plant, a phenomenon known as **vivipary** (e.g., *Rhizophora mangle*, Fig. 18-6). The germinated seeds are eventually shed, drop into the water like a sword, are attached to the muddy substratum, and continue to grow. The causal factors behind vivipary are not fully understood. ABA is involved because the endogenous ABA content of these seeds is often, although not always, quite low (about 25–50% of that usually

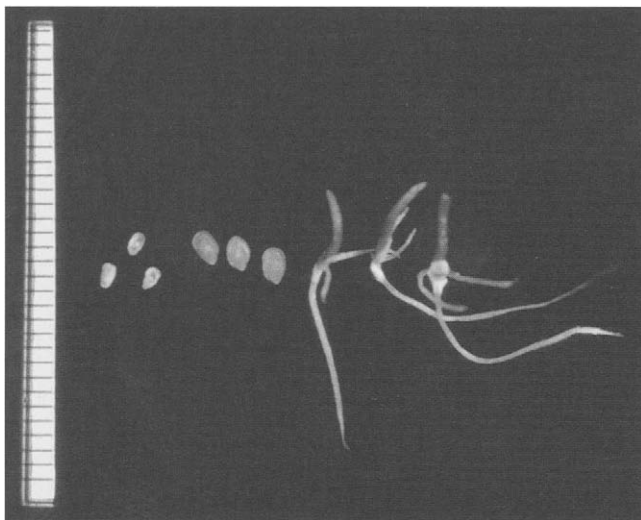


FIGURE 18-5 Culture of zygotic embryos \pm ABA. Wheat embryos (1.5mm in length) were excised from the grains (and mother plant) at mid-late stages of embryogenesis. (Left) At the beginning of incubation; (middle) after 5 days in culture +100 μ M ABA; (right) after 5 days in culture without ABA. Note that the embryo and scutellum continue to enlarge in ABA. From Quatrano *et al.* (1983).



FIGURE 18-6 Red mangrove (*Rhizophora mangle*) plants grow in intertidal waters. The seeds germinate and grow to lengths of 20–25 cm while still on the mother plant. Note a cluster of seedlings with long sword-like roots. Eventually, they fall and become planted in the mud below.

found in seeds). Moreover, application of ABA curtails vivipary to some extent, although not altogether, because insensitivity to ABA also seems to be involved.

Similarly, seeds of ABA-deficient mutants of tomato (e.g., *sitiens*) and *Arabidopsis* (e.g., *aba*) germinate readily when placed in water and sometimes show vivipary (the *aba* mutant referred to in this Chapter is *aba3*; see ABA-deficient mutants in Fig. 10-8, Chapter 10). If ABA is supplied to embryos of these mutants excised at midembryogenesis, normal development/maturation follows. For ABA-deficient *vp* mutants (e.g., *vp2*, *vp5*, *vp7*, *vp9*) of maize, the situation is more complicated. In some, viviparous growth is arrested by ABA and normal maturation follows, whereas in others, insensitivity to ABA seems to be operative, a situation similar to that in embryos of some mangrove plants.

Fluridone treatment inhibits carotenoid biosynthesis and thus reduces endogenous ABA synthesis. Wild-type maize kernels treated with fluridone germinate readily when placed in water and, in some cases, show vivipary, effects that are countered if ABA is given simultaneously.

These data from embryos in culture, from seeds of mangrove plants, from ABA-deficient mutants, and from fluridone treatment suggest strongly that ABA has a role in the prevention of premature germination during normal seed development. This conclusion is illustrated schematically in Fig. 18-7. Maturation and quiescence are shown as terminal events in a normal developmental program that embryos at a certain stage enter if ABA is available in sufficient concentration or if they are placed in a solution of high osmoticum. In the absence of ABA or high osmoticum, they continue with the germination program and do not complete the maturation program.

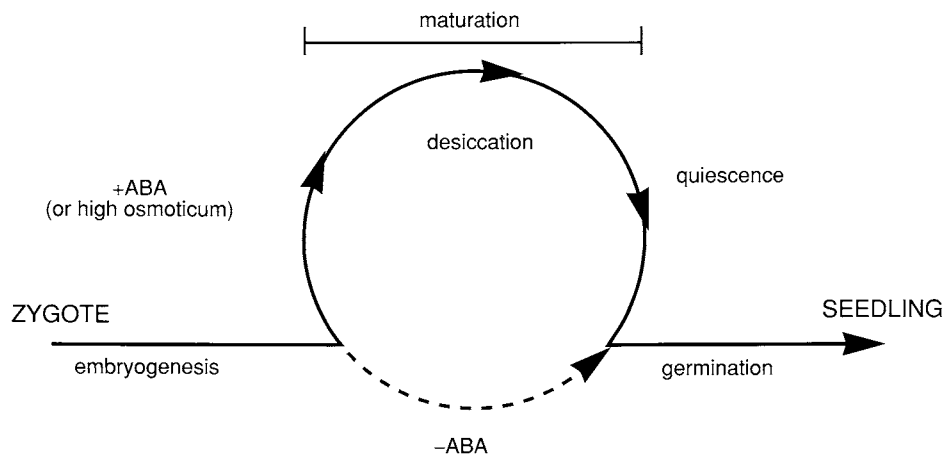


FIGURE 18-7 A general scheme connecting embryo development, maturation, and germination. The maturation “loop” is entered in the presence of ABA or high osmoticum; in the absence of ABA or osmoticum, embryos continue on the germination program. Modified from Rock and Quatrano (1995).

4.2. Desiccation Tolerance

In early stages of seed development, before ABA concentrations rise, protection against desiccation damage is probably afforded by sugars and other low molecular weight solutes. The longer term capacity of seeds to withstand desiccation, however, is dependent on ABA and is gradually built up in the developing seeds. If embryos are excised from seeds at early stages of development and dried by being placed in either warm air or a solution of high osmolarity, they are not able to cope with desiccation—they die, but as they mature, especially after reserve food deposition is more or less complete, they are able to withstand desiccation to much lower moisture contents. Clearly, some changes occur in the developing embryos that prepare them for withstanding the dry state.

The chief factor in desiccation tolerance appears to be the synthesis of late embryogenesis-abundant (or LEA) proteins (see Chapter 16). LEA proteins are not storage proteins nor do they have any specific role in seed dormancy. Rather, they are proteins that have a role in the protection of macromolecules and membranes both from crystallization due to loss of water and from high salt concentration, although precisely how they do so is still unclear. This role is supported by the activation pattern of *LEA* genes during seed imbibition and germination. *LEA* transcripts are preserved in the dry seed and are degraded soon after germination is completed. In seeds that have been temporarily hydrated, however, *LEA* genes can be activated transcriptionally.

There is considerable evidence that ABA induces the synthesis of LEA proteins during seed development. This synthesis occurs over a considerable time—some being synthesized as early as the beginning of the expansion phase and others later, but the highest LEA concentrations occur at the start of desiccation. This period coincides roughly with the rise and peak in ABA content. Isolated embryos in culture synthesize LEA transcripts and proteins on being supplied with ABA. A specific subset of LEA proteins is induced in desiccation-intolerant barley embryos cultured for 5 days in ABA, during which time the embryos acquire desiccation tolerance. Many *LEA* genes, e.g., the *Em* gene in wheat, contain nucleotide sequences in their promoters, which specifically respond to ABA (see Chapter 23).

4.3. Other Stress-Related Proteins

Potentially dangerous reactive oxygen-free radicals can be produced as by-products of respiration during late embryogenesis when water content is low. These

oxygen radicals can also be produced in hydrated but dormant seeds and in early stages of seed germination. These free radicals are scavenged by antioxidant enzymes, such as superoxide dismutases (SODs), catalases, and glutathione S-transferases (GSTs). cDNA clones and genes encoding isoforms of SODs are known from maize, and transcripts of several of them (e.g., *Sod3.2*, *Sod3.3*, and *Sod3.4*) are induced by ABA during embryo development. Corroborative evidence comes from the ABA-deficient *vp5* mutant, where exogenously supplied ABA induces the accumulation of *Sod3.2*, *Sod3.3*, and *Sod3.4* transcripts.

In seeds of barley and *Arabidopsis*, a protein, *PER1*, with sequence similarity to a group of antioxidants, named peroxiredoxins, is synthesized in late embryogenesis. The gene encoding *PER1* has been expressed in *Escherichia coli*, and the fusion protein reduces oxidative damage to DNA *in vitro*.

Other stress-related proteins include heat shock proteins (HSPs). The cDNA clone of a low molecular weight HSP has been isolated from mRNA extracted from mature sunflower seeds. Stage-specific hybridizations showed that the transcripts were maximally present in the embryo during the midmaturation stage and in the dry seeds.

4.4. ABA Regulation of Synthesis/Accumulation of Seed Food Reserves

While the roles of ABA in preventing precocious germination and induction of desiccation tolerance are well substantiated, its role in the accumulation of seed food reserves is still unclear. (For readers unfamiliar with storage reserves, it might be useful at this juncture to read Appendix 3.)

In whole seeds, the evidence is mainly restricted to the correlation that the highest amounts of ABA are present during the most active phase of seed enlargement and deposition of food reserves. While this coincidence is well documented (e.g., in seeds of wheat, rape, soybean, french bean), it does not mean causality. More direct evidence comes from isolated embryos grown in culture \pm ABA (see Section 4.1). Thus, for excised embryos of rapeseed (*Brassica napus*), the synthesis of napin and cruciferin requires the presence of ABA in the culture medium; if ABA is withdrawn, the synthesis of napin mRNA ceases. Similar data are available for the synthesis of a 7S globulin (an embryo-specific protein), the wheat germ agglutinin (WGA, a lectin), and the *Em* protein (a member of LEA D19 group) in the isolated embryos of wheat.

Other reports cast doubt that ABA has much to do with the accumulation of seed protein reserves. In some instances, the deposition of seed protein pre-

cedes the increase in ABA content (e.g., wheat germ agglutinin in wheat), or the supply of ABA has no effect on the accumulation of protein reserves in excised embryos (e.g., deposition of legumin in *Pisum sativum* embryos). Still other studies show no correlation between an increase or a decrease in ABA and storage protein accumulation. Thus, an increase in endogenous ABA content by four fold has no effect on sucrose synthase activity or zein accumulation in cobs of corn grown under water deficit, and a reduction in ABA by fluridone treatment of seed pods of alfalfa has no effect on storage protein synthesis.

Evidence from ABA-deficient or -insensitive mutants is not as clear as one would like. In ABA-deficient mutants of *Arabidopsis* and tomato (*aba* and *sitiens*, respectively), the deposition of storage food materials remains unaffected. However, it has been argued that these mutants may be leaky and still produce enough ABA to induce storage protein synthesis. In the ABA-deficient mutant *vp5* of maize, embryos homozygous for the allele do not accumulate the embryo-specific globulins GLB1 and 2, but do so when ABA is supplied to embryos *in vitro*.

Storage protein deposition in the ABA-insensitive mutants, *abi1* and *abi2*, of *Arabidopsis* (for ABA-insensitive mutants, see Chapter 23) is little affected. However, in seeds of the *abi3* mutant, a locus that regulates genes that are more seed specific than the other two, only two-thirds as much protein and about one-third the amount of eicosenoic acid (the major fatty acid of lipid reserves) accumulate compared to the wild type. The ABA-insensitive mutant *vp1* of maize does not accumulate the globulins GLB1 and 2 in the embryo *in vivo*.

Genes encoding many seed storage proteins have been cloned and sequenced. Analyses of the promoter sequences of these genes have revealed common *cis* sequences that confer seed-specific expression (e.g., the prolamin box in cereals; legumin and vicilin boxes for legumin and vicilin genes in dicots). *cis* Sequences specific for ABA-induced gene expression, ABA response elements (ABRE), have also been identified in many storage protein genes, such as that for helianthinin. Some genes may have one whereas others have two ABREs, but not all storage protein genes have an ABRE. Thus, an analysis of *cis* sequences has not yielded any conclusive evidence in favor of an ABA-induced synthesis of storage protein genes (for more details on ABA-induced gene expression, see Chapter 23).

If ABA is involved in the synthesis of storage proteins, might it also be expected to be involved in the synthesis of other storage reserves, such as starch and lipids (triacylglycerols). To date, though, there are no reports about ABA induction of any of the major

enzymes associated with either starch synthesis or synthesis of triacylglycerols (see Appendix 3). There are reports, however, of ABA inducing the synthesis of an oleosin, a protein associated with lipid bodies, and, as mentioned before, napin and cruciferin (two storage proteins) in isolated embryos of rapeseed in culture.

In conclusion, while the rise in ABA undoubtedly coincides with the accelerated deposition of food materials in seeds, and several lines of evidence support the idea that ABA is the inducing stimulus, conclusive proof is lacking. The possibility exists that the accumulation of at least some reserve foods is independently regulated and that ABA makes such accumulation possible by maintaining the developmental program of seeds.

4.5. Induction of Seed Dormancy

ABA has long been associated with dormancy, mainly because the hormone can be detected in both developing and mature seeds and is known to be inhibitory to germination, when applied exogenously. Direct evidence for its role in induction of dormancy, however, comes from ABA-deficient or -insensitive mutants.

4.5.1. ABA-Deficient and -Insensitive Mutants

Freshly harvested seeds of wild-type tomato and *Arabidopsis* do not germinate and are dormant, whereas those of ABA-deficient or -insensitive mutants germinate readily. In *Arabidopsis*, the ABA-deficient and -insensitive double mutant (*aba abi3*) shows precocious germination and vivipary. Thus, lack of endogenous ABA or ABA function correlates with lack of seed dormancy. Moreover, some mutants that are exceptionally sensitive to ABA (e.g., *era* mutants in *Arabidopsis*, see Chapter 23) are even more dormant than the wild type.

Genetic crosses between wild-type and ABA-deficient mutants in tomato and *Arabidopsis* indicate that it is the ABA content of the embryo, not that of the maternal tissues, such as seed coat, that determines the induction of dormancy in embryos (Fig. 18-8). In Fig 18-8, data are plotted according to vivipary and show clearly that a lack of ABA in embryo correlates with enhanced vivipary. There is a negative correlation between vivipary and dormancy. Vivipary is, in fact, an exaggerated example of lack of dormancy where seeds germinate while still on the mother plant.

Measurement of the endogenous ABA content of seeds of the wild-type (*Sit/Sit*) tomato (or the embryo/endosperm of the heterozygous mutant, *Sit/sit*) indicates that the ABA content peaks a few weeks after pollination and is not detectable afterwards. These

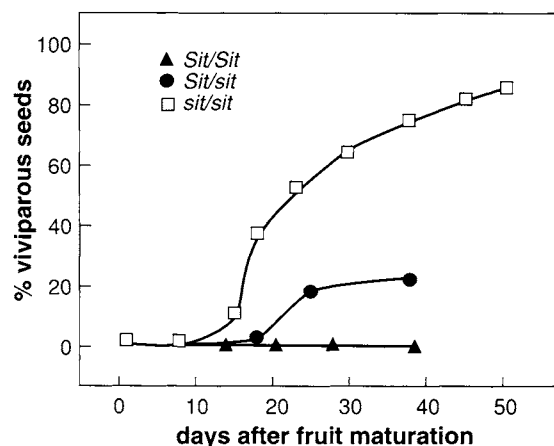


FIGURE 18-8 Percentage of seeds germinated in ripe fruit of tomato derived from self-pollination of *Sit/Sit* (homozygous wild-type), *Sit/sit* (heterozygous), and *sit/sit* (homozygous ABA-deficient) plants. Maturation is defined as the moment the fruit turns red. From Groot and Karssen (1992).

data imply that ABA is needed for a certain duration, but is not continuously required for the induction of dormancy.

4.5.2. Curtailment of Vivipary by Application of ABA

Although dormancy is induced during development, it becomes manifest only when seeds are incubated for germination. This makes it difficult to devise experiments that test for dormancy induction by application of ABA while the seed is still developing. Accordingly, some authors have used a curtailment of viviparous or precocious germination after ABA application as a test for dormancy. These experiments have utilized seed pods (in legumes) or cob sections from maize that can be manipulated after excision from the mother plant. Thus, sections of corn cobs containing kernels were placed in fluridone solution \pm ABA. Those with ABA showed dormancy in $> 50\%$

kernels, but only if such treatment was given between 13 and 15 days after pollination (DAP); kernels transferred at 16 DAP, or later, remained nondormant and indeed could be viviparous (Table 18-1).

In summary, the genetic evidence from tomato and *Arabidopsis* mutants indicates that ABA is involved in the induction of seed dormancy and that ABA comes from embryonic sources. It also suggests that ABA is required only for a specific period during seed development. Data from the application of fluridone \pm ABA to excised cobs or seed pods also suggest that the ABA content during a specific window in seed development is important for the induction of dormancy. This time seems to be about midembryogenesis. These data explain why the ABA content in mature, dry seeds does not necessarily bear a relationship to seed dormancy.

5. ENVIRONMENTAL CONDITIONS DURING SEED DEVELOPMENT AFFECT THE ACQUISITION AND EXTENT OF DORMANCY

Environmental conditions, particularly temperature and relative humidity (RH), during the latter half of seed development affect the acquisition and extent of dormancy. In temperate grasses, such as wheat, barley, and wild oat, lower temperatures are reported to enhance dormancy. The effect of relative humidity was shown by moving potted wheat plants at 35 days postanthesis (DPA) for 15 days to "wet," humid conditions (90–100% RH) or to "dry" conditions (35–40% RH) and subsequently testing them for germination (Fig. 18-9). Seeds from plants kept under dry conditions took a significantly greater number of days to reach 50% germination, i.e., they were more

TABLE 18-1 Effects of ABA on Vivipary in Maize Kernels^a

Age at ABA treatment (DAP)	Total number of kernels	Viviparous (%)	Dormant (%)	Viable (%)
13	21	43	57	100
14	19	37	63	100
15	16	19	81	100
16	15	100	0	NA ^b
17	17	100	0	NA
18	37	100	0	NA

^aSections of maize cobs with kernels were cultured with fluridone (100 mg liter⁻¹) and transferred to a medium containing fluridone and ABA (10⁻⁴ M) at ages 13 to 18 DAP. From Hole *et al.* (1989).

^bNot applicable.

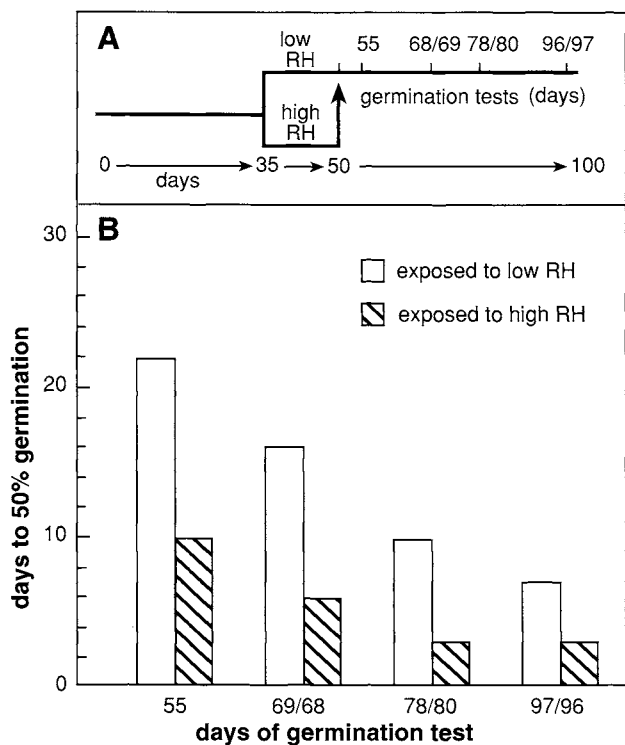


FIGURE 18-9 Effect of relative humidity (RH) on dormancy of wheat grains. Wheat plants were grown in pots in the greenhouse under defined conditions of day length and temperature. Normal grain maturation/desiccation in wheat begins at about 40 days postanthesis (DAP). At 35 DAP, pots were divided into two lots. One lot was kept at low humidity (RH 35–40%, “dry” ears), whereas the other lot was transferred to conditions of high humidity (RH, 90–100%, “wet” ears). After 15 more days, the latter batch (wet ears) was also transferred to low humidity conditions. From 55 to 97 DAP, batches of 40–50 grains (from four to five ears) were tested for germination on wet filter paper in petri dishes. (B) Days to 50% germination are shown by the histograms; the greater the number of days to germination, the deeper the dormancy. (A) The experimental protocol. Modified from King (1993).

dormant, than those given a wet treatment during the critical 35 to 50 DPA. In the “wet” ears, some grains even showed vivipary. Brief (4-day) drying episodes during the critical period could prevent sprouting and induce some dormancy, but only if given before 50 DPA. Day length has also been reported to affect dormancy. In *Chenopodium album*, long days during seed maturation enhance dormancy, whereas seeds maturing under short days are almost nondormant.

6. ABA AND SUSCEPTIBILITY OR RESISTANCE TO PREHARVEST SPROUTING

Preharvest sprouting in cereals is a special form of vivipary that occurs in mature dry grains, still held on

the ear, when they become wetted by rain or high humidity. In crops such as wheat and *Sorghum*, it is a source of considerable loss to farmers. It has a genetic and hormonal basis. Cultivars of cereal crops differ in regard to the potential for preharvest sprouting; some are very susceptible, others are highly resistant, and still others are in between. The effects of temperature seem to vary; in some cultivars, lower temperatures and a higher relative humidity promote preharvest sprouting, whereas in others, warmer temperatures and high humidity promote it. Moreover, populations of grains within the same cultivar vary in their sprouting behavior (Fig. 18-10), which suggests intrapopulation variations in sensitivity thresholds to water availability and/or responsiveness to ABA.

Responsiveness to ABA has been studied for cultivars that are susceptible or resistant. Embryos of sprouting-susceptible cultivars have a marginally, but probably not significantly, lower ABA content than those of resistant cultivars, but the major difference is in their sensitivity to ABA. If excised embryos are germinated in the presence of ABA, susceptible cultivars are much less responsive to inhibition of germination by exogenous ABA than resistant ones (Fig. 18-11).

7. “RECALCITRANT” SEEDS

As mentioned earlier, many seeds remain hydrated at maturity and are damaged easily if dried. In tropical species, they may also be damaged by exposure to chilling temperatures (e.g., 5°C, see chilling injury, Chapter 16). In nature, these seeds go on to germinate after being shed. They usually cannot be stored for more than a few months, and then too with special precautions about temperature and relative humidity. Mangrove plants are a special example of recalcitrant seeds, but the phenomenon is much more widespread and is seen in many temperate and tropical forest, or in plantation, trees and shrubs, e.g., species of oak (*Quercus*), horse chestnut (*Aesculus*), maple (*Acer*), walnut (*Juglans*), rubber (*Hevea*), cacao (*Theobroma*), and coffee (*Coffea*). It has a genetic basis in that some species of a genus or cultivars of the same species may produce recalcitrant seeds, whereas others may produce orthodox seeds (e.g., *Acer rubrum* produces orthodox seeds, whereas *A. saccharum* produces recalcitrant seeds).

In some cases, it has been possible to induce tolerance to desiccation if drying is done under carefully controlled conditions. However, the biochemical basis



FIGURE 18-10 Preharvest sprouting in wheat (*Triticum aestivum*) results from insufficient embryo dormancy and is prevalent in seasons with cool, damp autumns or heavy rainfall around harvest time. The three ears on the left have been induced to sprout by placing plants of cultivar, Boxer, under cool, moist conditions during the later stages of grain growth. The three ears on the right are from plants of the same variety grown under identical conditions, but show no visible signs of sprouting, indicating intrapopulational variability. Courtesy of John Lenton, BBSRC, UK.

for recalcitrance is still unclear. Measurements of endogenous ABA generally indicate a lower content in embryos of recalcitrant seeds compared to their orthodox counterparts. The reports on LEA proteins are controversial. Some authors have reported that they are absent. Others have indicated that the LEA subgroup, dehydrins, is present in embryos of some recalcitrant species (e.g., *Quercus robur*). Identifications of LEA proteins based on hybridizations using a cDNA clone or immunoblots using antibodies prepared against a conserved sequence of dehydrin proteins may not distinguish a functional from a nonfunctional dehydrin. Thus, while some recalcitrant species produce LEA-type proteins, the proteins could be defective and unable to function as long-term protectants. Alternatively, the appropriate LEA proteins may not be present.

8. HARDENING OF THE SEED COAT AND OTHER CHANGES

Many structural and biochemical changes occur in the cell walls, membranes, and vacuoles of seeds in later stages of embryogenesis, prior to or accompanying desiccation. These include lignification of the outer

cell walls of the seed coat in some seeds, deposition of cuticle and waxy substances, deposition of phenolic substances in vacuoles, and possibly changes in membrane phospholipids and proteins. These changes have been little investigated, and it is not clear whether the stimulus for these changes comes from ABA or some other factor. Deposition of various defense-related lectins, α -amylase inhibitors, and antifungal proteins is somewhat better known. Lectins and α -amylase inhibitors are deposited along with reserve proteins (see Appendix 3); antifungal proteins are deposited in the outer cell layers and probably serve to discourage fungal attack during storage and early stages of seed germination.

9. DESICCATION MAY ACT AS A SWITCH TO TERMINATE THE DEVELOPMENTAL AND TURN ON THE GERMINATIVE PROGRAM

After a certain stage of embryo development, seeds, or isolated embryos, can be dried prematurely without killing the embryo. In these prematurely dried seeds, the expression of several storage protein genes (e.g.,

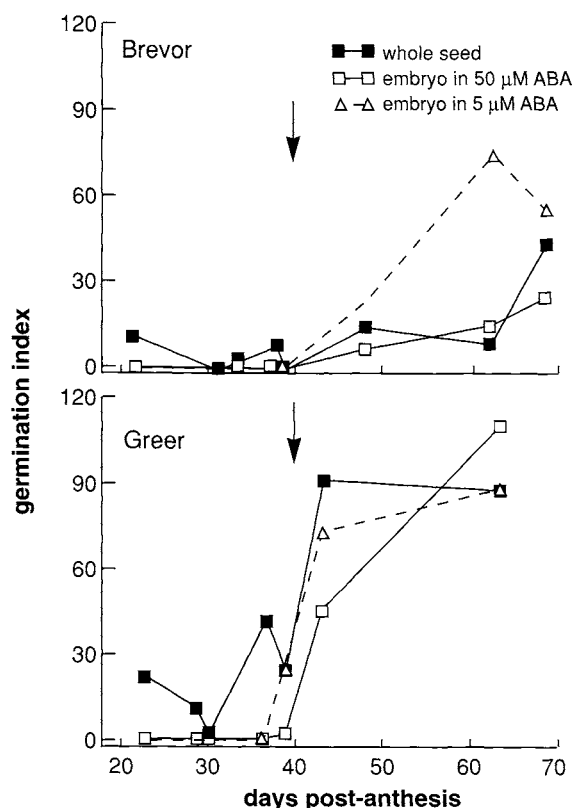


FIGURE 18-11 Germination indices for excised embryos from two cultivars of wheat; sprouting-resistant cv Brevor and sprouting-susceptible cv Greer. Isolated embryos were incubated in 5 or 50 μ M ABA. Whole seeds of cv Greer show much more sprouting than those of cv Brevor. In parallel, excised embryos of cv Greer are much less susceptible to inhibition of germination by exogenous ABA than the excised embryos of cv Brevor. Arrows indicate the time when whole seeds attained maximum fresh weight. Each data point represents 10 embryos. From Walker-Simmons (1987).

phaseolin in bean; ricin D and a lectin in castor bean; medicagin in alfalfa) is terminated, and their transcripts, if any are left, are destroyed quickly and are not resynthesized upon seed hydration and germination. Instead, the genes for enzymes involved in the mobilization of reserve starch (e.g., α -amylase), proteins (e.g., protease, carboxypeptidase), and fatty acid utilization (e.g., malate synthase, isocitrate lyase) are turned on. These genes are characteristically expressed after seed germination when the storage reserves are utilized; their mRNAs are either absent from or present in very low amounts in the developing seed. This has led to the suggestion that, in orthodox seeds, maturation drying acts as a switch that terminates the developmental program, including deposition of food storage, and turns on the germinative program to commence under favorable conditions (Fig. 18-12).

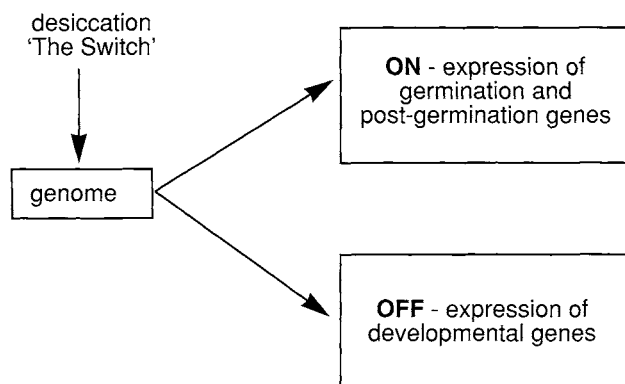


FIGURE 18-12 Consequences of desiccation on the expression of genes encoding developmental or germinative/post germinative proteins in seeds. From Bewley (1995).

10. SEED MATURATION AND DORMANCY AFFECT SEED VIABILITY AND SUBSEQUENT GERMINATION

As explained in the foregoing sections, species vary as to whether they produce seeds that become quiescent after completion of development, as in most orthodox species, or must germinate very quickly after being shed, as in recalcitrant species. In the former category, seeds may also acquire dormancy, as in most noncultivated species. Among the species that produce dormant seeds, different seed lots vary considerably with respect to their dormancy. These variations are often the result of environmental conditions during seed development/maturation, especially the latter half when desiccation tolerance and dormancy are acquired. Fully mature and dormant seeds survive well in the soil over long periods of time (average is ~ 3 –5 years in soil, if not eaten by insects, worms, birds, or rodents). They also store well and germinate to give rise to healthy seedlings. In contrast, seeds that are not fully dormant spoil easily in storage and do not germinate well under normal germination conditions. Dormancy, however, is not a prerequisite for survival. Desiccated and quiescent, but nondormant, seeds also survive well in dry storage, although not in soil. "Seed vigor" is a term that is sometimes used to describe seed or seed lot quality. Vigorous seeds are those that give rise to healthy, sturdy seedlings, whereas nonvigorous seeds give rise to weak, sickly looking seedlings. These variations among different seed lots of the same species are of obvious importance to seed centers, gene banks, nurseries, and forest-related industries.

11. CHAPTER SUMMARY

Seed development in flowering plants proceeds in three discrete, although continuous, stages: early, mid-, and late embryogenesis. In early embryogenesis, there is extensive cell division activity; also, the body plan of the embryo is laid down and endosperm development begins. Midembryogenesis is a period of intense and varied biochemical activity. The embryo and, in many seeds, endosperm expand and reach their final sizes. Food reserves, such as storage proteins, lipids, and polysaccharides, are elaborated from photoassimilate that is translocated to the seed and deposited *in situ* in endosperm and/or embryonic tissues. Most seeds also accumulate calcium and magnesium salts of phytic acid, which provides seeds with reserves of phosphorus (see Appendix 3). Proteins that serve to protect cell membranes and macromolecules against the injurious effects of dehydration, such as LEA proteins, antioxidants, and heat shock proteins, are also synthesized. In addition, many seeds deposit toxic substances such as lectins, phenolics, and an amylase inhibitor, which prevent pathogen attack as well as discourage predation and herbivory. These synthetic activities may continue into the third phase, late embryogenesis, but eventually stop as the seeds lose water and desiccate. Additionally, some changes in proteins and/or other macromolecules occur, the nature of which is still obscure, which allow the seed/embryo to become quiescent and, in some seeds, also dormant. The maturing seeds acquire a hard, resistant seed coat and, in the dry state, have a negligible metabolism and respiration rate.

Four hormones play major roles in seed development. IAA and cytokinin concentrations are high in early embryogenesis when cell divisions occur at a high frequency. IAA also plays a role in patterning and polarity establishment in the young embryo. The role of gibberellins is not as clearly defined, but they seem to be involved in expansion growth of the embryo and endosperm, thus creating a sink for photoassimilates. ABA content rises when the concentrations of the other three hormones have declined or are declining. ABA plays a central role in several events that occur in mid- to late embryogenesis. It is responsible for the induction of desiccation tolerance and, in some seeds, of dormancy as well. It is believed responsible for the synthesis/accumulation of at least some reserve proteins. Most of all, it seems to maintain the embryo in a developmental program mode during the activities, just given. After these inductions or events are completed, the free ABA content in most seeds drops to a low or insignificant amount. Desicca-

tion of seed seems to act as a switch, terminating the developmental and maturation program and turning on the germinative program, which requires rehydration of the seed and, in some species, breaking of dormancy.

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1. INTRODUCTION

Seed germination is defined as the sum of events that begin with hydration of the seed and culminate in emergence of the embryonic axis (usually the radicle) from the seed coat. Simple though this definition is, the details of radicle emergence from the seed coat or, its obverse, lack of radicle emergence despite favorable conditions (i.e., dormancy) are still not fully clear. Moreover, this definition applies mostly to seeds with well-developed embryos (e.g., pea, bean), where the breakdown of reserves to support seedling growth is primarily a postgermination phenomenon. In several

species, seed is shed when the embryo is still small and undeveloped (e.g., *Fraxinus*). The embryo continues to grow and differentiate within the seed, utilizing the food reserves in the endosperm, and protrusion of the radicle does not occur until much later. These seeds do not germinate while immature.

This chapter deals with changes in seeds associated with the resumption of embryo growth and development following hydration regardless of whether they are pre- or postgermination changes. The emphasis is on seeds of crop plants, such as legumes and cereals. Processes associated with the germination of seeds are outlined in Section I, followed by a consideration of seedling growth and mobilization of food reserves in Section II. Section III deals with seed dormancy, including dormancy associated with immature embryos.

SECTION I. SEED GERMINATION

1. IMBIBITION OF WATER

For seed germination to occur, the dry, quiescent seed must imbibe water and become hydrated; in addition, there is a requirement for oxygen (but see

below). The temperature requirements are more flexible. Germination occurs over a wide range of temperatures, although for each species there is an optimal temperature and the rate of germination drops off at both above and below that temperature. Assuming no barriers to hydration (e.g., the presence of an impervious seed coat), the initial uptake of water is rapid (phase I) and is followed by a plateau (phase II) (Fig. 19-1). Metabolic reactivation of seeds starts immediately on imbibition and is closely associated with the rise in respiration rate and production of ATP.

Almost all seeds are capable of some anaerobic respiration in the early hours of imbibition, but the seeds of some plants that grow in flooded soils with low oxygen tension [e.g., rice (*Oryza sativa*), barnyard grass (*Echinochloa phyllopogon*), *Typha latifolia*, *Juncea effusus*)] are capable of germination, even some seedling growth, under anaerobic conditions. These seeds show considerable activities of alcohol dehydrogenase (ADH) and lactate dehydrogenase (LDH), enzymes involved in the fermentation of pyruvate produced during glycolysis to ethanol or lactate, respectively. For continued growth and development, however, an oxygen supply is necessary, and, with its provision, pyruvate is shunted toward oxidative decarboxylation in mitochondria and the activities of ADH and LDH decline.

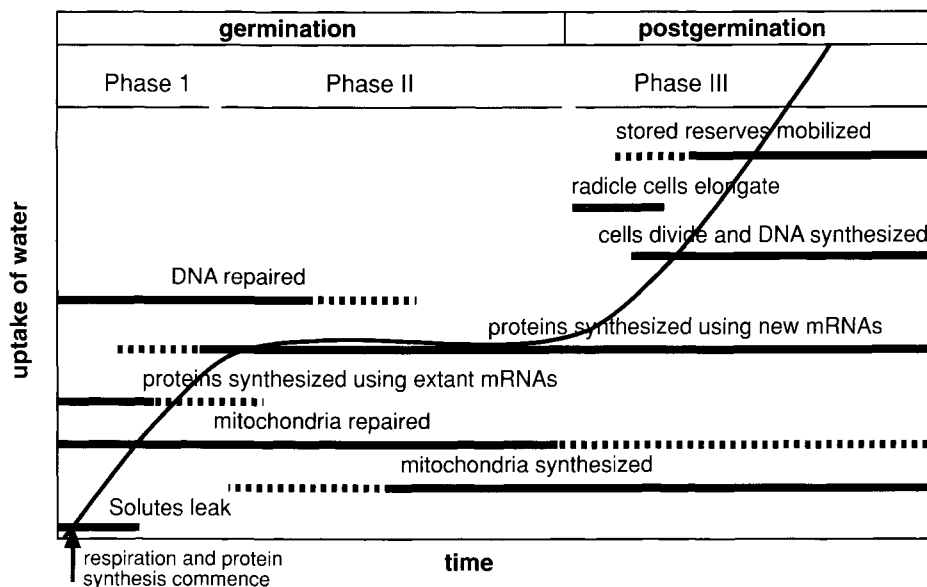


FIGURE 19-1 Time course of major events associated with seed germination and seedling growth. The uptake of water by the dry, quiescent seed is shown in three phases: rapid at first (phase I) and then a plateau (phase II), followed by a second rise coincident with the beginning of seedling growth (phase III). The time for events to be completed varies from hours to many weeks, depending on the plant species and the germination conditions. The respiration rate generally parallels the rate of water uptake. From Bewley (1997).

1.1. Metabolic Activation of Imbibed Seeds

In dry seeds, the respiration rate and metabolism are both extremely low, and membranes and various cell organelles are structurally ill-defined and biochemically inefficient. In the early hours of imbibition, membranes are leaky (the leakiness may be induced by the rushing in of water), and various low molecular weight substances, ions, sugars, and amino acids, leach out into the surrounding medium. Within a few hours of hydration, however, the membranes are restored to their normal liquid-crystalline structure and selective permeability. Other changes include restoration of organelles, such as mitochondria and nuclei, to their functions and replacement of single ribosomes by populations of polyribosomes as protein synthesis begins. Hydration, while allowing some loss of solutes (which may cause fungal growth), also results in loss of many inhibitors of germination, e.g., some phenolics and abscisic acid (ABA).

The rise in water content is paralleled by a rise in the respiration rate and O₂ consumption. Enzymes of the Krebs' cycle and the terminal oxidases are usually present in the dry seed and are reactivated, but new enzymes are also synthesized, as shown by the expression of both mitochondrial and nuclear genes encoding the subunits of cytochrome c oxidases. Such new synthesis of enzymes signifies biogenesis of new mitochondria, or repair to extant ones, which may occur almost exclusively in some species.

Protein synthesis in the early hours of imbibition generally involves translation of messages left over from development. These messages include transcripts for some LEA proteins and other stress-related proteins, such as heat shock proteins (HSPs) and antioxidants. If the water supply continues, these transcripts are gradually lost, but if it is interrupted, their genes may be reactivated. Other leftover transcripts include those for ribosomal proteins. Genes for these proteins become transcriptionally activated as germination proceeds.

Hydration of seeds not only restores metabolic activity to hitherto quiescent embryos, it also activates the embryonic machinery such that it can receive signals, such as light, chilling, and alternating warm and cold temperatures, signals that are involved in breaking certain types of seed dormancy (see Section III). Activated forms of hormones are also synthesized or converted from inactive precursors or conjugates on hydration.

1.2. Dormant vs Nondormant Seeds

Nondormant, imbibed seeds, as well as dormant, imbibed seeds, show very much the same set of

changes, as described earlier, during phases I and II, but whereas nondormant seeds go on to phase III to complete germination and seedling growth, and a fresh increase in water uptake and in respiration rate, dormant seeds never enter that phase. The reason for the difference, the fundamental question as to what makes a seed dormant, is still unanswered (see Section III).

2. RADICLE GROWTH AND PENETRATION OF TESTA

Elongation of the radicle and its emergence from the seed coat complete phase II of imbibition and germination. As discussed in Chapter 15, the elongation growth of stems and roots is driven by a combination of wall loosening and turgor pressure of constituent cells, and there is no reason to doubt that the same combination also applies to growth of the young radicle. However, despite much work, conclusive data are lacking. There is no clear correlation between radicle growth and osmotic potential (or solute concentration) in cells of the radicle, and although expansins, xyloglucan endotransglycosylases (XETs), and endo- β -1,4-glucanases (cellulases) are probably present, their involvement in radicle emergence has not been demonstrated.

The rupture of the seed coat (or tissues surrounding the embryo) in most cases occurs with pressure from the growing radicle tip. It is not known whether the hydrolysis of wall polysaccharides of the surrounding tissues (e.g., endosperm or perisperm) is required, although it probably occurs. In some species, which show coat-enhanced dormancy (e.g., tomato, *Datura* sp.), synthesis of cell wall hydrolyzing enzymes is reported.

Typically, cell division is not necessary for radicle emergence, but there are obvious exceptions in seeds with immature embryos. For subsequent growth, however, it is necessary.

2.1. Inhibitors of Seed Germination

The presence of free ABA in seeds or after uptake from the ambient medium inhibits seed germination. Seeds of different harvests vary in their ABA content and, in general, show a good correlation between their ABA content and their ability to germinate (Fig. 19-2). Also, seeds of ABA-deficient mutants germinate readily in water, but fail to do so if supplied with ABA. ABA-insensitive mutants also germinate readily or require much higher concentrations of ABA to inhibit germination than the wild type (Fig. 19-3).

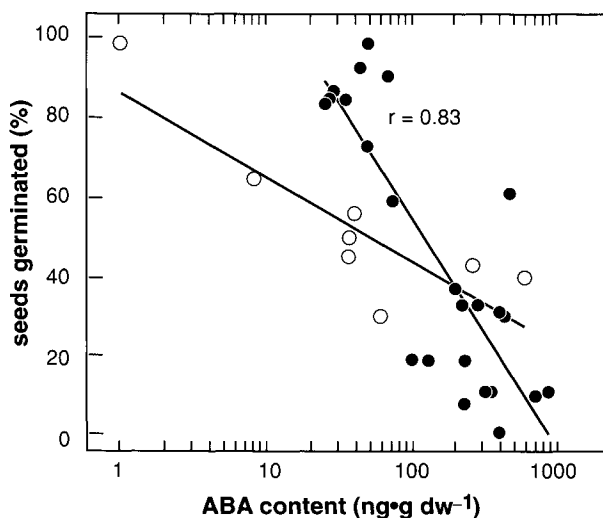


FIGURE 19-2 Correlation plot of ABA content in mature seeds of tomato (●) and *Arabidopsis* (○) against germination on water. From Hilhorst (1995).

ABA conjugates are present in dry seeds and may be hydrolyzed to yield free ABA with seed imbibition. As a result, the free ABA content may rise in some seeds in the early hours after the beginning of imbibition, especially if the seed coat is intact.

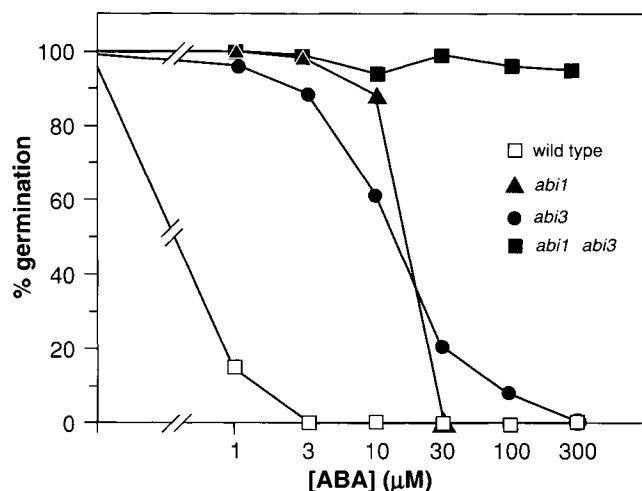


FIGURE 19-3 Effect of ABA on germination of seeds from wild type and two ABA-insensitive mutants (*abi*) of *Arabidopsis*. Data from *abi1* and *abi3* monogenic mutants, as well as the double mutant *abi1 abi3*, are shown; *abi1* mutation affects vegetative growth, as well as seed tissues, whereas *abi3* is more seed specific. Germination percentage was scored 4 days after plating on minimal media containing varying concentrations of ABA. Note that both mutants require much higher concentrations of ABA to inhibit germination by 50% than the wild type; the double mutant was very insensitive and showed nearly 100% germination at all concentrations of ABA. From Finkelstein and Somerville (1990).

The manner in which ABA inhibits seed germination is not understood. For some seeds (e.g., rapeseed, muskmelon seeds), it has been suggested that ABA prevents wall loosening or enhances the threshold for wall loosening in cells of the radicle, although a specific effect on any of the wall-loosening enzymes has not been demonstrated. Other suggestions include an interaction between endogenous ABA and water potential difference ($\Delta\Psi$) between the seed and the ambient medium. Apparently, the higher the potential difference, the lesser the sensitivity to ABA.

Other inhibitors of seed germination, besides ABA, include phenolic compounds, especially *trans*-cinnamic acid, coumaric acid, and coumarin, which are usually inhibitory at concentrations of $10^{-4}M$ or above. Seed coats of many plants deposit large amounts of phenolics as a defense against predation, and many of these phenolics are water soluble. They are released on imbibition and, if their concentrations are sufficiently high, they may cause inhibition, but how they do so is unknown.

3. SEED GERMINATION MAY BE MODULATED BY RELATIVE RATIOS OF ABSCISIC ACID (ABA) AND GIBBERELLINS (GA)

It has been suggested that seed germination is regulated by a balance between the relative amounts of endogenous GA and ABA in the seeds and sensitivities of seed tissues to these hormones. Evidence supporting this hypothesis comes from a study of seed germination in GA and ABA synthesis and/or response mutants in tomato and *Arabidopsis*. Seeds of GA synthesis mutants (e.g., *gib1* of tomato, *ga1* of *Arabidopsis*) require exogenous GA to complete their germination. Seeds of ABA synthesis mutants (e.g., *sitiens* in tomato, *aba* and *reduced dormancy* or *rdo* of *Arabidopsis*) germinate readily in water or require lower concentrations of exogenous GA for germination than seeds of the wild types. Similarly, ABA-insensitive mutants, which require much higher concentrations of ABA than the wild type to inhibit germination (see Fig. 19-3), germinate without exogenous GA or require lower doses of GA to induce seed germination. In contrast, GA-insensitive mutants (e.g., *gai*, *spy* of *Arabidopsis*) require lesser amounts of exogenous ABA to suppress seed germination. Whether these data from tomato and *Arabidopsis* are applicable to other seeds is unknown.

Many seeds (e.g., tomato, lettuce) have a resistant endosperm with galactomannan-rich cell walls, which the radicle is unable to penetrate. Endo- β -mannanases hydrolyze the mannan backbone of galactomannans and, along with two other enzymes, are responsible for

the breakdown of carbohydrate wall reserves during postgerminative seedling growth (see Section II, 3.2). In tomato, a small gene family of endo- β -mannanases has been identified. The mRNA of one isoform is expressed in the endosperm above the radicle tip during pregerminative imbibition. It is thought to cause weakening of the endosperm in that region (the endosperm cap), thus facilitating radicle penetration. The gene for this isoform is induced by gibberellins, which confirms earlier physiological and genetic data that GAs are required for the germination of tomato seeds. Interestingly, the expression of this GA-induced isoform is not inhibited by ABA. Other isoforms of endo- β -mannanases in tomato are expressed postgerminatively and are involved in the mobilization of wall reserves for seedling growth.

4. SECTION SUMMARY

Seed germination is a series of events that begin with imbibition and end with the emergence of the radicle from the seed coat. It includes the reacquisition by cell membranes of their selective permeability properties, repair and salvaging of DNA and other macromolecules damaged during desiccation, and restarting of the metabolic and synthetic machinery of the embryonic cells. Radicle emergence involves cell growth and rupture of the tissues surrounding the embryo, but typically does not involve cell division. Two hormones, gibberellins and abscisic acid, play contrasting roles in seed germination; gibberellins promote it, whereas ABA inhibits it. Not only are the relative ratios of endogenous GAs and ABA important, but also the sensitivities of seed tissues to these hormones seem to be involved. In tomato, a gene encoding an endo- β -mannanase has been identified. The gene is induced by GA and is expressed pregerminatively in the endosperm cap, but the expression of this gene is not inhibited by ABA. The manner in which these two hormones regulate seed germination is still very much an open question.

SECTION II. MOBILIZATION OF FOOD RESERVES

1. POSTGERMINATIVE CHANGES

The transition from phase II to phase III signals a shift in priorities from damage repair, metabolic reactivation, and emergence of the radicle to the rapid

growth of root and shoot and establishment of a green autotrophic seedling. Although a little of the stored food reserves may be hydrolyzed earlier in phase II, the bulk is mobilized in phase III after seed germination and provides substrates for energy, as well as building blocks for macromolecules. This heterotrophic period of nutrition lasts until emergence of the young shoot from the soil (or darkness) and the beginning of photosynthesis by the seedling. Growth of the young seedling is accompanied by extensive cell divisions and cell enlargement and requires abundant water and energy in the form of ATP. Thus, phase III begins with a fresh increase in rates of water uptake and respiration (see Fig. 19-1).

The new growth of root and shoot involves the transcription of new sets of genes, as some of the earlier sets are downregulated and cease transcription. Among the genes best studied during this phase are those encoding enzymes involved in the hydrolysis of stored food reserves. These genes have been particularly well studied in cereal grains, where many of them are expressed under the control of gibberellins. Mobilization of food reserves in dicot and conifer seedlings, especially the former, is also well documented, although the hormonal regulation of such mobilization is not clear. This section deals with these topics.

2. MOBILIZATION OF FOOD RESERVES IN CEREAL GRAINS

A seed is surrounded by a hard and often lignified seed coat (testa). In cereal grains, the testa is fused with the ovary wall (pericarp); hence, the seed is called a caryopsis. The endosperm occupies most of the seed and consists of two parts: parenchyma cells, which are loaded with starch and some protein and which are dead at maturity (the starchy endosperm), and an aleurone tissue (one to several layers) of living cells. The aleurone tissue, also called the aleurone layer, surrounds the starchy endosperm and the embryo.

2.1. GA Produced by the Embryo Is Responsible for Activation/Synthesis of New Enzymes

Hydrolysis of food materials is seen readily if barley grains are soaked in water for a few days. If the embryo is excised prior to, or within a few hours after, soaking, no such hydrolysis occurs. This phenomenon was known to Haberlandt, who speculated in 1890 that the embryo produces a factor that causes the aleurone layer

to release hydrolyzing enzymes. In the 1960s, L. Paleg in Australia and H. Yomo in Japan, independently, showed that GA supplied to deembryonated endosperm causes hydrolysis of starch, and Paleg further proposed that the GA produced by the embryo is responsible for such hydrolysis in the intact seed. Margaret Radley (1967) in England, working with wheat seeds, suggested that GA is synthesized first in the scutellum part of the embryo. In recent years, analysis of GAs in germinated grains of barley and wheat has revealed the presence of several GAs, including the biologically active GA₁. In intact but germinated grains of barley and rice, it has also been shown that hydrolytic activity spreads from the scutellum, outward into the endosperm. Furthermore, activity measurements in barley indicate that the hydrolytic enzymes appear first in the scutellum (1–2 days after beginning of imbibition) and later in the aleurone layer (peak around 3–4 days).

Together, these studies illustrate an elegant example of control over the utilization of stored food in some cereal grains (Fig. 19-4). The embryo produces GA, which moves to aleurone layer where it induces the production of various enzymes. The enzymes are secreted into the endosperm where the stored food is hydrolyzed, and the products of hydrolysis—sugars and amino acids—move back to the embryo to provide

for its growth. Thus, while the endosperm is the food bag, the key to the food bag rests with the embryo, its legitimate beneficiary. Moreover, the key (GA) does not have to be cut anew; as shown by the use of GA synthesis inhibitors, *de novo* biosynthesis of GAs from *ent*-kaurene is not required; active GAs are probably produced from inactive precursors.

2.2. Aleurone Layer or Aleurone Protoplasts Are Ideal for Studying a GA-Induced Response

Cereal grain is an excellent system to study a plant hormone response. The seed can be cut into two halves, one with and the other without the embryo, and the embryoless half can be used to study the induction of hydrolytic enzymes under the influence of exogenous GA. Alternatively, aleurone layers peeled from the half seed, or protoplasts prepared from the peeled layers, can be used. Isolated layers or protoplasts, although more difficult to prepare, provide a cleaner, more precise *in vitro* system to study the GA response. The aleurone layer is a homogeneous tissue consisting of only one cell type. No cell division or growth is involved. Also, the response is dramatic: the entire biochemical machinery of the cells

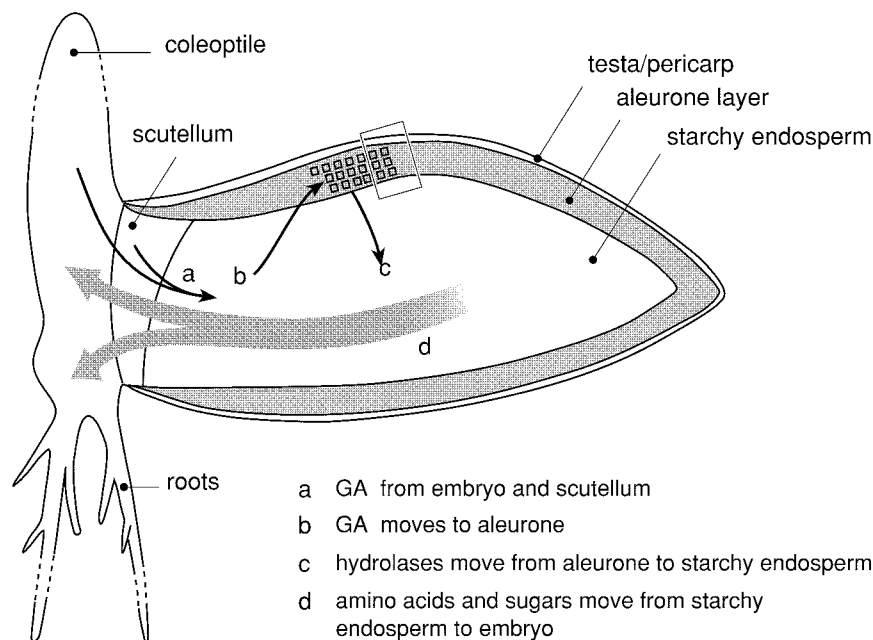


FIGURE 19-4 A sagittal section of a germinated grain showing the movement of GA from the embryo to the aleurone tissue (a and b) where the hydrolyzing enzymes are activated/synthesized, movement of enzymes to starchy endosperm (c), and hydrolysis of stored food and movement of hydrolyzed products from the endosperm to the embryo (d). The area enclosed by the rectangle represents roughly the area shown in Fig. 19-5A. From Jones and Armstrong (1971).

is changed as a result of GA treatment. Accordingly, a great deal of work on the molecular basis of gibberellin action has been done using aleurone layers or aleurone protoplasts. It should be noted, however, that while the basic response is similar, there are some differences in the kinetics of enzyme production between half-seeds, aleurone layers, and aleurone protoplasts (see Section II, 2.4). Aleurone layers or protoplasts of wheat, barley, rice, and oat are the favored materials for study. For reasons not very clear, aleurone cells of maize, sorghum, and certain cultivars of oat, wheat, and barley are relatively insensitive to added GA₃, which may have something to do with their relatively high endogenous GA content.¹

2.2.1. Structure of Aleurone Cells

The aleurone tissue is composed of living parenchyma cells; it is about three to four cell layers in barley and rice and one cell layer in wheat, oat, and maize. The cells have thick walls, which are rich in arabinans and arabinoxylans, but have little cellulose, and are interconnected by numerous plasmodesmata. Each cell is packed with protein bodies [protein storage vacuoles (PSVs), known as aleurone grains] with globoids embedded in them (Fig. 19-5A). Globoids, as mentioned elsewhere (see Appendix 3), are Ca²⁺ and Mg²⁺ salts of inositol hexaphosphoric acid (phytic acid). Other reserves include lipid droplets, sucrose, and some oligosaccharides, but there is little or no starch. Thus, aleurone cells are well equipped with quickly available energy sources (sugars and lipids), as well as with minerals and phosphorus. In the presence of gibberellins, hydrolytic enzymes are activated, or synthesized, the protein bodies lose their stored protein, and are replaced by numerous small vacuoles that later fuse to form one or two large vacuoles (see Figs. 19-5B and 19-5C).

¹The GA content of grains seems to vary with the cultivar, as well as temperature conditions at the time of grain maturation. Some species and cultivars produce abundant α -amylase without any exogenous GA treatment of half-seeds or isolated layers (e.g., maize, some cvs of barley and wheat). Others produce very low levels of background α -amylase and are assumed to have a low or negligible GA content (e.g., Himalaya barley, harvest 1985). (Actual measurements of GAs in grains or aleurone layers are very few and are not converted easily to fresh weight or volume of grain.) Also, within a cultivar, seeds maturing at a time when weather is warm produce more α -amylase, which suggests that they have more endogenous GA than those maturing in cooler weather. Note that this correlates with dormancy which is less in wheat and barley grains exposed to warmer than to cooler temperatures during the latter half of their development. Researchers usually select a cultivar and a particular seed lot that does not have much endogenous GA in the aleurone tissue and stay with it.

2.2.2. Aleurone Cells and Scutellar Epithelial Cells Are Secretory Factories

While the aleurone layer is endospermic in origin, the scutellum is an embryonic tissue. The scutellum is surrounded by an epithelial layer, which is similar to the aleurone tissue in wall characteristics and the nature of stored reserves. Cells of the aleurone layer and scutellar epithelium, in an activated state, have a well-developed endomembrane system for the synthesis, processing, and export of numerous proteins. The hydrolysis of lipid reserves provides fatty acids that are used for membrane synthesis and also as fuel for energy. The hydrolysis of proteins stored in PSVs provides amino acids, which serve as building blocks for the synthesis of new proteins. PSVs are the first targets of proteases and endopeptidases produced by these tissues.

Some of the newly synthesized enzymes are active at the site of production, i.e., in the cells of aleurone tissue and scutellar epithelium. Others are packaged in vesicles and are secreted into the apoplast from

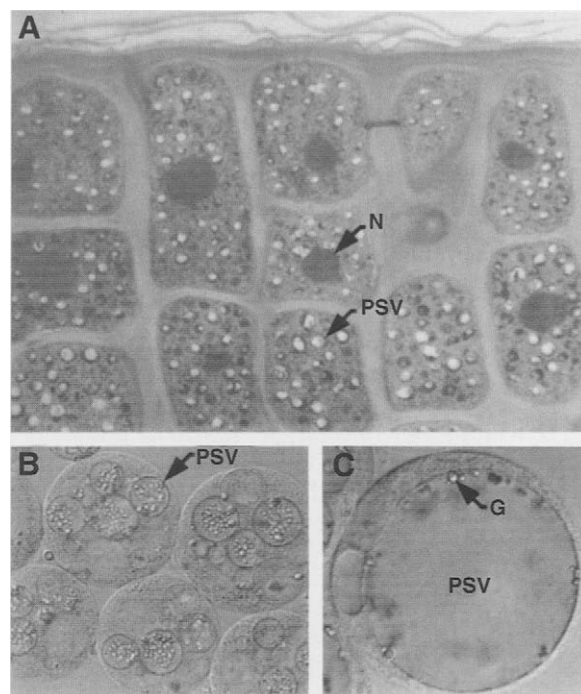


FIGURE 19-5 Photomicrographs of a barley aleurone layer and aleurone protoplasts. (A) Transverse section through the aleurone layer. The seed coat and fruit wall are fused in the caryopsis and are impossible to separate, but the two together peel off readily from the starchy endosperm. Each cell has a prominent nucleus (N) and numerous protein storage vacuoles (PSV). (B) A protoplast from an aleurone cell at an early stage after GA₃ treatment; numerous PSVs are still present. (C) A protoplast at a later stage of GA₃ treatment with one large vacuole. A phytin globoid is still visible (arrow). From Bethke *et al.* (1997).

where they diffuse to the starchy endosperm. The relative contributions of scutellum vs aleurone tissue in the provision of hydrolytic enzymes for the starchy endosperm in intact seeds *in vivo* are not known. Generally, it is thought that the scutellum contributes in early stages following germination, and its contribution is relatively small (see also Section II, 2.4). The following account deals with aleurone tissue, although generally it is also applicable to the scutellum.

2.3. Protein Synthesis in Aleurone Layers

Protein synthesis *in vivo*, as well as cell-free translation of extracted RNA, using [35 S] methionine and gel electrophoresis shows that GA affects the activity/synthesis of many proteins in the aleurone layer, some are upregulated, whereas others are downregulated (Fig. 19-6). Among the upregulated proteins, one type of protein, α -amylase, is preeminent, accounting for up to 30% of new protein synthesis. α -Amylase is an endo- α -1,4-glucanase, which breaks the glucan backbone in amylose and amylopectin and provides oligomers for further action by other glucanases (e.g., β -amylase, limit dextrinase). ABA applied at the same time as GA, or very shortly after, inhibits the action of GA and, at a high enough concentration, has some effects of its own (see Section II, 2.7).

2.3.1. Enzymes Expressed or Activated by GA in Aleurone Tissue

In the aleurone tissue, GA affects the synthesis/activation of many different enzymes/enzyme complexes. Some of the important enzymes are listed in Table 19-1 (see later in this chapter) to illustrate the range of enzymes expressed.

2.3.2. α -Amylases Are a Large Family of Enzymes

α -Amylases are produced in abundance and are among the most studied enzymes in plants. They are encoded by multigene families, which show tissue and developmental specificity in their expression. α -Amylases present in vegetative parts (e.g., leaves, stems) and vegetative storage organs (e.g., tubers) differ in their expression patterns and inducing stimulus from those present in seeds. Even in seeds, α -amylases expressed during seed development/maturation differ from those expressed following seed germination. Electrophoretic and ion-exchange chromatography of α -amylases produced following germination in cereal grains reveal two major groups of isoenzymes; one group has its isoelectric point (pI) between pH 5.9 and 6.6 (the high pI group) and the other has its pI between 4.4 and 5.2 (the low pI group) (Fig. 19-7).

Members of each group are encoded by their respective genes, which differ in their kinetics of expression, as well as sensitivities to GA concentrations.

2.3.3. Kinetics of α -Amylase Accumulation

In barley aleurone layers, the mRNAs of low pI α -amylases are constitutively present, but with application of GA increase in amount over a period of about 48 h. High pI α -amylase mRNAs are synthesized *de*

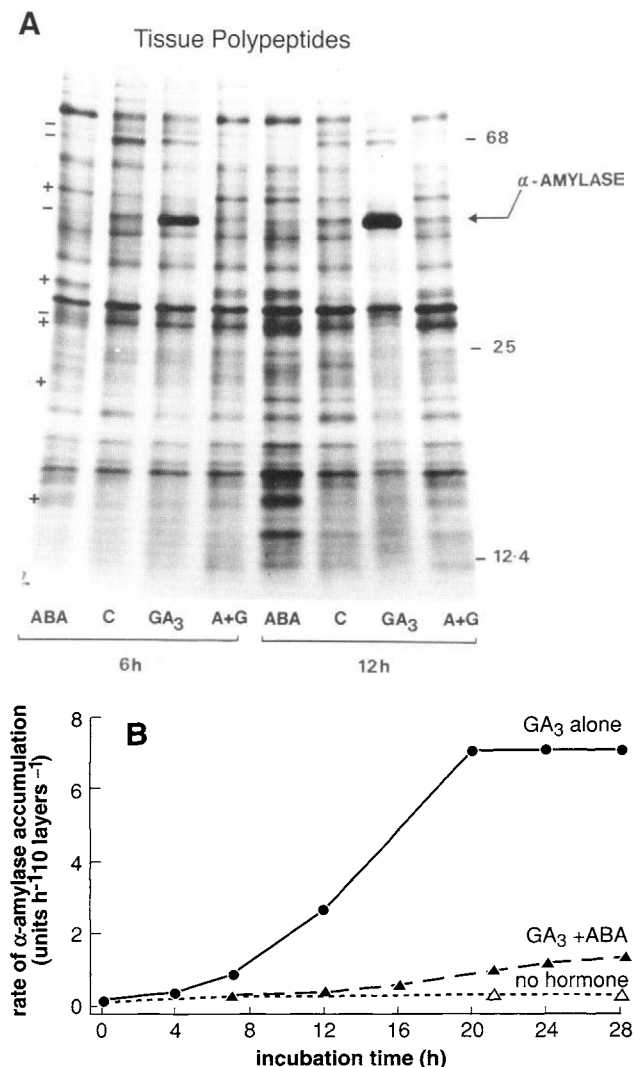


FIGURE 19-6 (A) Gel electrophoresis of *in vivo* [35 S]methionine pulse-labeled proteins extracted from barley aleurone layers after GA and/or ABA treatment for 6 and 12 h. The most prominent band after GA treatment is that of α -amylase. Minus and plus signs refer to polypeptides that were decreased or increased, respectively, by ABA. Molecular weights in kilodaltons are shown on the right, C, control, no hormone. (B) Rates of accumulation of α -amylase protein in isolated aleurone layers and incubation medium in response to exogenous GA, ABA, and GA and ABA in combination. From Jacobsen and Chandler (1987) with kind permission from Kluwer.

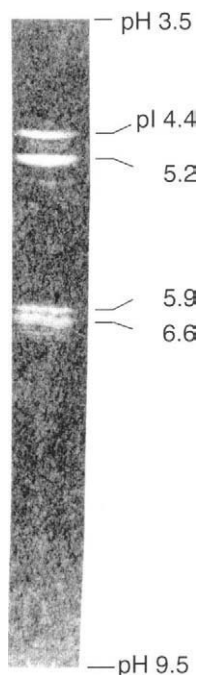


FIGURE 19-7 α -Amylases expressed in barley grains after germination fall into two groups based on their isoelectric points: low pI group (4.4–5.2) and high pI group (5.9–6.6). From Jacobsen and Chandler (1987) with kind permission from Kluwer.

novoo, reach peak production at 12–16 h, and then decline (Fig. 19-8). The periods of maximal production of α -amylase proteins correspond with the periods of maximal mRNA expression. Similar kinetics are known for low pI and high pI groups of α -amylases in wheat and rice.

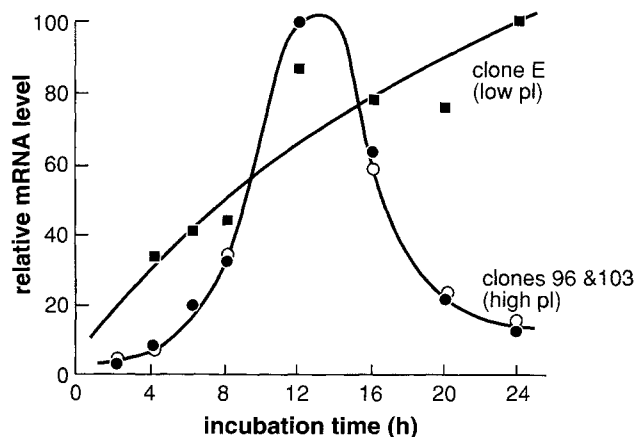


FIGURE 19-8 Kinetics of accumulation of low pI and high pI α -amylase mRNAs in aleurone layers of barley. cDNA clones specific for high pI or low pI α -amylases were used to study the kinetics of accumulation using Northern hybridization. From Jacobsen and Chandler (1987) with kind permission from Kluwer.

The two groups of isozymes also differ in the concentration of GA required for their induction. mRNAs of low pI α -amylases are induced at considerably lower GA concentrations (1 nM) than mRNAs of high pI α -amylases (100–1000 nM) (Fig. 19-9), a situation also seen for some genes encoding ethylene-induced enzymes, e.g., ACC synthase vs polygalacturonase (see Chapter 17). This mechanism could provide an explanation of how one hormone can regulate the activity of many different genes.

2.3.4. Structure of α -Amylase Genes in Cereals

α -Amylase genes are highly conserved, with about 428 to 440 amino acids and with long stretches of homology, but they diverge at three locations; the carboxy terminus, the signal peptide (for insertion into endoplasmic reticulum), and a small stretch around amino acid 280, termed the α -amylase signature region (Fig. 19-10). The homology between members of one group among wheat, barley, and rice is much greater than the homology between high pI and low pI genes in the same genus, which suggests that the two groups of α -amylase genes separated before the evolution of barley, wheat, and rice. The number, position, and orientation of introns in the α -amylase genes are also invariant.

2.3.5. Other GA-Induced or -Repressed Genes in Aleurone Tissue

GA-induced responses in aleurone tissues of wheat, barley, rice, and oat have been studied intensively since the mid-1980s, and numerous clones of cDNAs and genes encoding many other proteins, in addition to α -amylases, have been obtained. They include clones for cysteine proteinases, carboxypeptidases, (1 \rightarrow 3, 1 \rightarrow 4)- β -glucanases, α -glucosidases, and an RNase; for barley, in which most work has been done, it is possible to correlate their order of appearance following GA treatment. Lipid-digesting enzymes, some endo- and exopeptidases, and some wall-digesting enzymes [e.g., xylanases, (1 \rightarrow 3, 1 \rightarrow 4)- β -glucanases] make their appearance before the α -amylases. α -Amylases are unable to penetrate the cell walls; hence, to reach their target cells in the endosperm, it is necessary to digest the cell walls.

Several other enzymes are downregulated by GA treatment. These include the α -amylase inhibitor, which is stored in the dry seed as a protection against insect predators, alcohol dehydrogenase for use in anaerobic respiration, some HSPs, and so on.

2.4. Gibberellin Action in Whole Seeds

Isolated aleurone layers and aleurone protoplasts have provided a wealth of information on the regula-

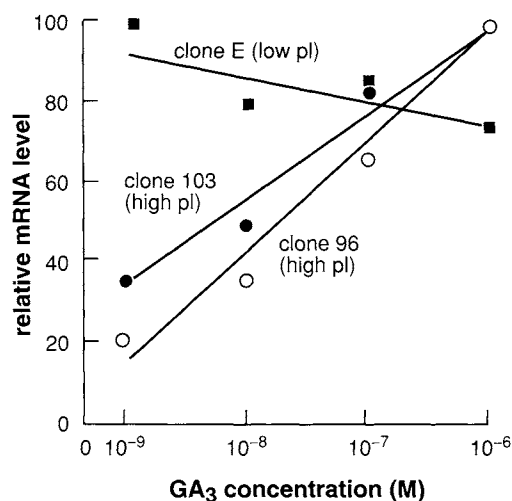


FIGURE 19-9 GA concentration dependence of induction of low *pI* and high *pI* α -amylase genes in barley aleurone layers. RNA was isolated from aleurone tissue treated with different GA concentrations for 12 h. RNA was extracted and probed with gene-specific cDNA clones. From Jacobsen and Chandler (1987) with kind permission from Kluwer.

tion of GA-induced gene expression, as well as sites of GA perception and signal transduction (see Chapter 24) that would have been difficult to obtain with intact, germinated seeds. Nonetheless, a few aspects of the gibberellin response in cereal grains require work with whole grains. The role of the scutellum in providing hydrolytic enzymes has been reinvestigated. Transcripts for a $(1 \rightarrow 3, 1 \rightarrow 4)$ - β -glucanase in barley appear first in the scutellum and subsequently in the aleurone layers. Similar results are reported for an α -amylase gene in rice (Fig. 19-11). Does the synthesis of hydrolytic enzymes in the scutellum also require

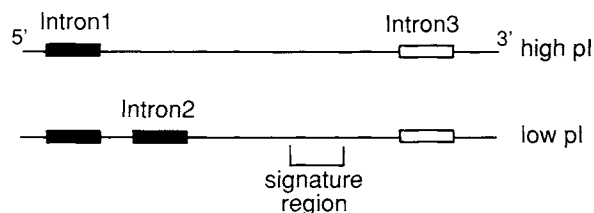


FIGURE 19-10 Diagrammatic maps of high *pI* and low *pI* α -amylase genes in barley. The genes have two or three introns; introns 1 and 2 are close together and near the 5' end, whereas intron 3 is close to the 3' end. Both high and low *pI* genes have introns 1 and 3, but only low *pI* genes have intron 2. The signature region is so designated because it contains small localized insertions, which sort the genes into subfamilies. Because insertions are rare, genes with the same insertion are believed to have descended from a common gene and therefore belong to the same group. Modified from Jacobsen *et al.* (1995) with kind permission from Kluwer.

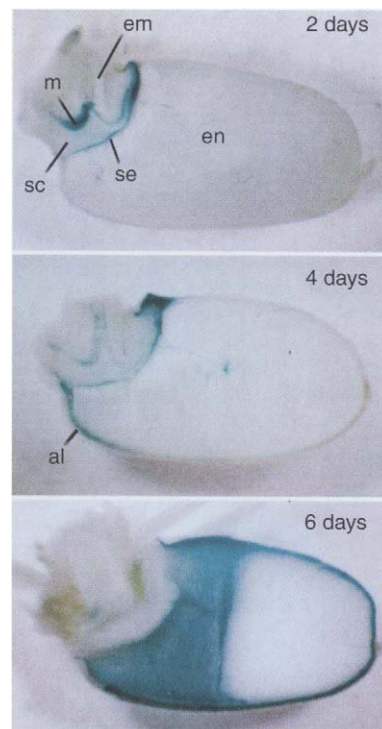


FIGURE 19-11 Expression of an α -amylase gene in rice seeds. Rice plants were transformed with a construct consisting of the promoter of a rice α -amylase gene (*RAmy1*) and the coding sequence of the reporter gene, *GUS*. Transgenic seeds were germinated in water for 2, 4, and 6 days at 30°C, cut in half longitudinally, and stained for glucuronidase activity. *GUS* activity first appeared in scutellum on day 2 and peaked at day 4. In the aleurone layer, *GUS* activity was very low on day 2, increased on day 4, and peaked on day 6. em, embryo; m, mesocotyl; sc, scutellum; se, scutellar epithelium; en, starchy endosperm; al, aleurone layer. From Itoh *et al.* (1995).

endogenous GAs? The answer is yes because a GA-deficient mutant of barley requires exogenous GA for the synthesis of enzymes.

An interesting observation deals with the kinetics of induction of high *pI* and low *pI* α -amylases. It will be recalled that in isolated aleurone layers, low *pI* mRNA is constitutively present in low amounts but increases to high amounts in 24 h and does not decline until after 48 h, whereas high *pI* mRNAs are synthesized *de novo* and reach a peak at 12–16 h and then decline. In intact seeds, the situation is reversed; high *pI* mRNAs appear first, are dominant at 2–3 days, and then decline, whereas low *pI* mRNAs begin to accumulate after 3 days and decline only after 7 days (Fig. 19-12). These data suggest that some factor(s) present in whole seeds but absent from isolated aleurone layers is also involved in the regulation of α -amylase genes and emphasize caution in extending results from isolated systems *in vitro* to intact whole organisms.

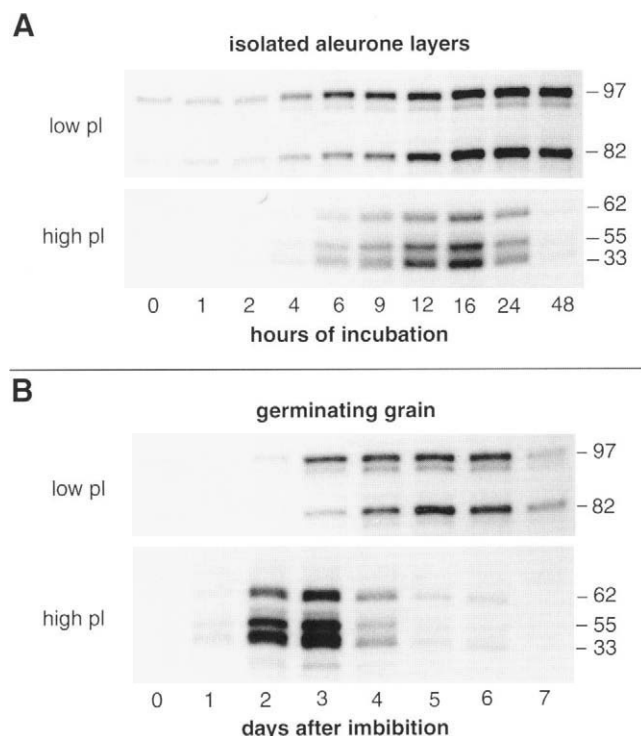


FIGURE 19-12 Expression of high pI and low pI α -amylase genes in aleurone layers (A) and intact germinating seeds (B), both given $1 \mu\text{M}$ GA_3 . Numbers on right refer to molecular mass (kDa). From Jacobsen *et al.* (1995) with kind permission from Kluwer.

2.5. Secretion of Hydrolases and Regulation of Their Activity

Gibberellins not only regulate the synthesis/activation of many hydrolases, they are also thought to be responsible for the maintenance of an active secretory process and for the maintenance of an acidic environment in the endosperm, which is required for optimal activities of many of these hydrolases.

2.5.1. Maintenance of an Active Secretory Apparatus

Elsewhere, we have seen how nascent polypeptides are processed in the endomembrane system and packaged in Golgi vesicles for transit to various organelles or *via* the default pathway to the cell exterior (see Appendix 3). Many of the enzymes synthesized *de novo* under the control of GA are also processed in the ER and Golgi network. Many are glycosylated before export. A high concentration of free cytosolic calcium, $[\text{Ca}^{2+}]_{\text{cyt}}$ (referred to as Ca^{2+}), has been implicated in various processes where vesicular traffic to the plasma membrane is involved. One of the best examples is tip growth in pollen tubes where exocytotic vesicles laden with nascent wall materials fuse with the tip and provide not only wall but also mem-

brane materials for the growing pollen tube. This traffic is regulated by a steep concentration gradient of Ca^{2+} , high at the tip and low toward the pollen grain, which is maintained by an inwardly directed Ca^{2+} import from the external medium across the tip (see Chapter 2). Russell Jones at U. C. Berkeley, California, and associates have shown using both barley aleurone layers and isolated protoplasts that, on addition of GA_3 to the incubation medium, the free Ca^{2+} concentration in the cytosol of aleurone cells rises from about 100 nM to about 500 nM within a few hours, before any secretion of hydrolases. Use of calcium fluorescent dyes further shows that most of the free Ca^{2+} is focused near the plasma membrane, which is necessary for vesicular fusion and exocytosis (Fig. 19-13). In the absence of Ca^{2+} from the incubation medium, the secretory activity is inhibited, possibly because of reduced membrane trafficking. This effect of GA on elevation of cytosolic Ca^{2+} is negated by ABA.

Russell Jones' group has also shown that GA_3 induces the synthesis of a Ca^{2+} -ATPase located on the ER membrane, which actively pumps cytosolic Ca^{2+} into the ER. Such accumulation is necessary for α -amylase processing in the endomembrane system.

2.5.2. Maintenance of an Acidic Environment

Many of the enzymes that hydrolyze proteins and starch, as well as cell wall-digesting enzymes that are active in the aleurone layer and starchy endosperm, have acidic pH optima. The cereal endosperm is acidic at imbibition because of a high concentration of malic acid. The amounts of malic acid decline as germination proceeds, but an acidic pH environment is maintained because gibberellin action involves an enhanced secretion of phosphoric and citric acids from the aleurone layer. This acidic environment is conducive to a lowering of the pH of PSVs in the aleurone cells (from 6.6–7.0 to 5.8 or lower) and the activation of proteases and phosphatases with an acidic pH optima, which in turn hydrolyze the stored proteins and phytate.

The pH optima for hydrolases, such as $(1 \rightarrow 3, 1 \rightarrow 4)$ - β -glucanases and α -amylases, that catalyze the degradation of cell wall and starch, respectively, are around pH 4.5. The β -amylase and limit dextrinase that assist α -amylase in starch degradation require proteolytic cleavage for activation. While β -amylase is present in the endosperm, limit dextrinase is secreted from the aleurone layer (see Table 19-1). The endopeptidases responsible for this cleavage are GA induced, secreted to the endosperm, and have acidic pH optima. In addition, the transport of amino

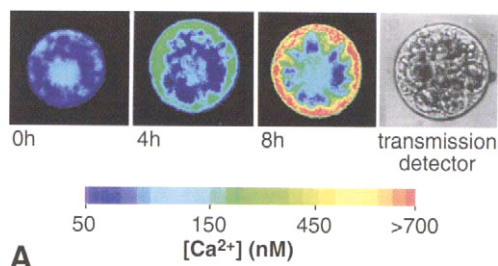
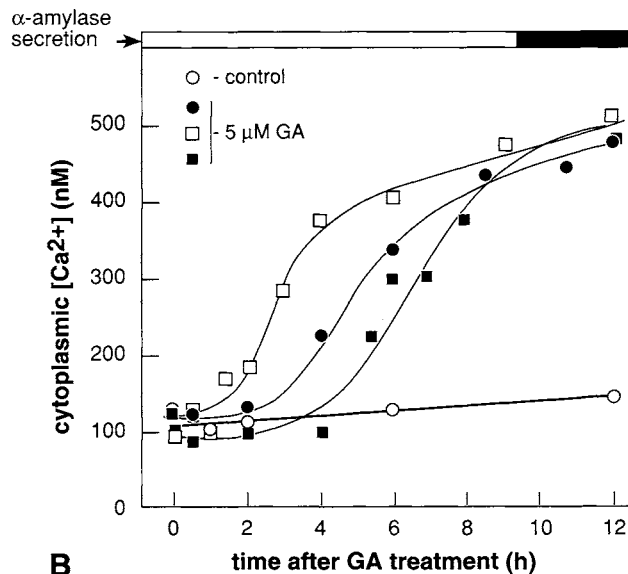


FIGURE 19-13 Cytoplasmic Ca^{2+} content and secretory activity in barley aleurone protoplasts. (A) Cytosolic Ca^{2+} distribution in protoplasts treated with $5\ \mu\text{M}$ GA_3 for 0, 4, and 8 h, and a transmission detector picture of a protoplast taken 24 h after GA treatment. Ca^{2+} , as indicated by fluorescence, is present in high concentrations near the cell periphery. (B) Representative time course of changes in average cytosolic Ca^{2+} concentration in single aleurone protoplasts either not treated (control) or treated with $5\ \mu\text{M}$ GA_3 (three replicates are shown). Bar at the top shows the time course for α -amylase secretion. Amounts of cytosolic Ca^{2+} were determined by microinjecting indo-1-dextran (a fluorescent calcium-binding dye) and ratio imaging under a confocal laser-scanning microscope at indicated times after GA treatment. From Gilroy (1996).



acids and sugars from the endosperm to the embryo,-breakdown of inhibitors such as α -amylase inhibitor, and solubilization of otherwise inaccessible substrates for hydrolytic action are probably all favored by an acidic environment.

2.6. End Product Inhibition of α -Amylase Production and GA Synthesis by the Embryo

The expression of α -amylase genes reaches a peak, but then the mRNA levels decline, although they may

TABLE 19-1 GA -Induced Enzymes in Cereal Aleurone Tissue^a

Function	Enzyme	Synthesized <i>de novo</i>	Secreted
Phospholipid synthesis	Phosphocholine esterase	<i>b</i>	No
Phosphorous metabolism	Phosphatases (e.g., phytase, ATPase)	?	Yes
Digestion of cell wall	(1-3, 1-4)- β -Glucanase	Yes	Yes
	Xylanases	Yes	Yes
	Arabinases	?	Yes
Hydrolysis of lipids	Isocitrate lyase	?	No
	Malate synthase	?	No
Hydrolysis of proteins	Cysteine proteinases	Yes	Yes
	Carboxypeptidases	?	Yes
Hydrolysis of starch	α -Amylases	Yes	Yes
	α -Glucosidases	Yes	Yes
	β -Amylases	<i>c</i>	
	limit dextrinase	<i>d</i>	Yes
Hydrolysis of nucleic acids	RNases	Yes	Yes

^aCompiled from Rogers and Rogers (1999), Tibbot *et al.* (1998), Shintani *et al.* (1998), Banik and Fincher (1996), and Jones and Jacobsen (1991).

^bExpressed constitutively, but amount or activity is enhanced in the presence of GA .

^cActivated by proteolytic cleavage *in situ* in the endosperm.

^dActivated by proteolytic cleavage *in situ*; also synthesized *de novo* in aleurone and secreted to the site of action.

fluctuate for a while. Reasons for the decline are not very clear, but a negative feedback control by the end product has been suggested. Sugars are the products of starch hydrolysis, and, among them, glucose is the most abundant, accounting for more than 50% of the total soluble sugars in rice and barley. In whole seeds, sugars and amino acids are used for embryo growth, but in isolated aleurone layers, sugars are likely to accumulate and inhibit α -amylase production. In addition to negative control by the end product, the presence of sugars external to aleurone layers may impose an osmotic stress on the aleurone cells, which in turn may inhibit protein synthesis generally. Some data also suggest that an increased sugar concentration in the endosperm acts as a negative regulator for GA biosynthesis in the embryo. How these regulations of either α -amylase genes or genes for GA biosynthesis are effected is unknown.

2.7. ABA Inhibition of GA-Induced Gene Expression and Secretory Activity

In the aleurone layer, all genes that are upregulated by GA are also downregulated by ABA. In addition, several genes that are downregulated by GA, e.g., α -amylase inhibitor and alcohol dehydrogenase, are upregulated by ABA. In addition, as mentioned earlier, ABA also counters the GA-induced rise in cytosolic Ca^{2+} and inhibits the secretory process. How does ABA counter these GA effects? We do not know. It is likely that the interaction occurs at some common point(s) where the GA signaling and ABA signaling pathways intersect or share a common domain (see Chapter 24).

3. MOBILIZATION OF FOOD RESERVES IN DICOTS AND CONIFERS

3.1 Mobilization of Food Reserves

Large amounts of proteins, lipids, and starch are accumulated in seeds of dicots and conifers during seed development and maturation. In some plants, hemicelluloses, especially mannans and galactomannans, are deposited in the cell walls of storage organs (see Appendix 3 and Table A3-3). These reserves are mobilized, and the products are used as building blocks for macromolecules and as fuel for energy during seedling growth. As a result, the fresh weight of the embryonic axis and plumule increases, whereas that of cotyledons (or endosperm or megagametophyte) falls, and the amounts of stored foods decline (Fig. 19-14).

3.2. Enzymes and Their Genes

Enzymes involved in mobilization of these reserves in dicots are well identified and, in many cases, are better characterized than similar enzymes in cereal grains. Lipids (triacylglycerols) are hydrolyzed by lipases to yield fatty acids and glycerol. Fatty acids are used for membrane synthesis and for gluconeogenesis *via* β oxidation and the glyoxylate cycle (Fig. 19-15). The glyoxylate cycle is discussed in greater detail in Chapter 20. Genes encoding lipases, as well as isocitrate lyase (ICL) and malate synthase (MS), two key enzymes in the glyoxylate cycle, have been cloned from several plants and their mRNAs shown to be expressed in germinated embryos (e.g., rapeseed, cucumber, castor bean), endosperm (e.g., castor bean), or megagametophytes of germinated pine seeds.

Several proteases (e.g., cysteine endopeptidases, aspartic proteases, other endo- and exopeptidases) and α -amylases have also been isolated from a number of plants and their genes cloned.

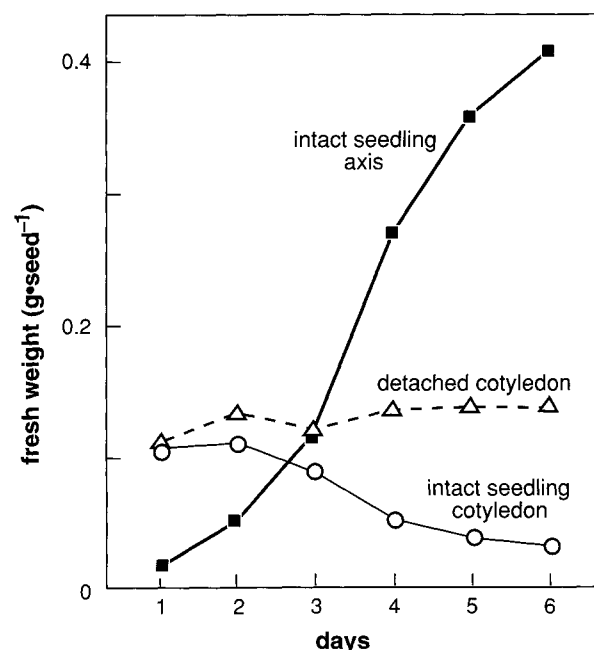


FIGURE 19-14 Changes in fresh weight of germinated or incubated organs of *Phaseolus mungo* seeds. After 7 h of initial imbibition, seeds were divided into two groups. In one group, intact seeds were allowed to germinate at 26–28°C in the dark, and, at 1-day intervals, cotyledons and axis organs were separated and weighed. In the second group, cotyledons were detached from the embryonic axis and incubated under the same conditions. As is evident, in intact seedlings, the fresh weight of the embryo axis increases, whereas that of cotyledons falls; in detached cotyledons the weight remains the same or increases slightly. From Minamikawa (1979).

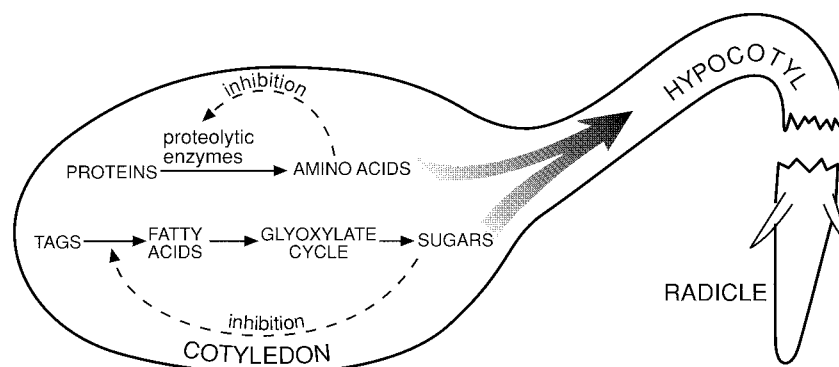


FIGURE 19-15 Mobilization of reserve proteins and triacylglycerols (TAGs) in cotyledons of cucumber. Feedback inhibition of proteases and lipases by their products is also shown. From Bewley and Black (1994) with kind permission from Kluwer.

Some of these enzymes are stored in the dry seed (e.g., β -mannosidase in lettuce), whereas others are synthesized *de novo* from amino acids released by the hydrolysis of stored proteins (e.g., ICL in rapeseed and pine, lipase in castor bean, an endopeptidase in mung bean, an α -amylase in pea cotyledons, endo- β -mannanases in lettuce and tomato).

The digestion of galactomannans in cell walls of lettuce endosperm has been particularly well studied. Digestion requires the action of three enzymes: an endo- β -mannanase cleaves the mannan backbone into oligomeric fractions, an α -galactosidase cleaves the galactose side chains from the mannan oligomers, and a β -mannosidase hydrolyzes the oligomers to mannose monomers (Fig. 19-16A). It is thought that GAs (and/or cytokinins) from the embryonic root-shoot axis move *via* cotyledons to the endosperm where they bring about the *de novo* synthesis of endo- β -mannanase and α -galactosidase. The oligomannans and galactose residues released by the action of these two enzymes move from the endosperm into the cotyledons where the oligomannans are further broken down into mannose residues by the action of β -mannosidase (Fig. 19-16B). The products of hydrolysis, galactose and mannose residues, are converted to sucrose and utilized for seedling growth.

In some legume and other seeds, where galactomannan deposits in cell walls of endosperm are so extensive that the cells die (e.g., fenugreek, date and ivory nut, see Table A3-3, Appendix 3), an aleurone-like layer surrounding the dead tissue synthesizes and secretes the three wall hydrolases. In other endospermic legumes (e.g., carob) and in date palm (*Phoenix dactylifera*), where the deposits may not be so heavy, the endosperm cells are metabolically active after germination and are the source of hydrolases.

3.3. Role of Embryo or Embryonic Axis on Reserve Food Mobilization

Does the embryo exercise any control over the mobilization of food reserves in the major storage organs, cotyledons or endosperm? One way to answer this question is to surgically isolate the root-shoot axis and plumule from the cotyledons or, alternatively, isolate the whole embryo from the endosperm, incubate the two separately, and monitor the mobilization of reserves and/or changes in enzyme activities.

In one such study on mung bean cotyledons, in intact seedlings, the total reserve protein dropped by 75% in 6 days, accompanied by a rise in the activity of extractable proteinase. The amounts of soluble (or free) amino acids in cotyledons declined after 3 days, presumably because they were transported to the embryonic axis. In contrast, in detached cotyledons, there was very little drop in protein content, a little increase in proteinase activity, and an increase in soluble amino acids after 3 days, presumably because they could not be utilized by a sink (embryonic axis) (Fig. 19-17, see also Fig. 19-14).

Many other studies have also shown that the embryonic axis (or the whole embryo) exercises a positive control over the mobilization of reserves in cotyledons (or endosperm) and activities of various hydrolyzing enzymes, but contradictory data showing no effect of the axis on enzyme activity are also known, sometimes from the same species (e.g., pea).

3.4. Hormonal Regulation of Hydrolytic Activities

If the embryonic axis (or embryo) indeed regulates the mobilization of reserves in cotyledons (or endosperm or megagametophyte), how does it do so? The parallel from the cereal aleurone layer suggests that

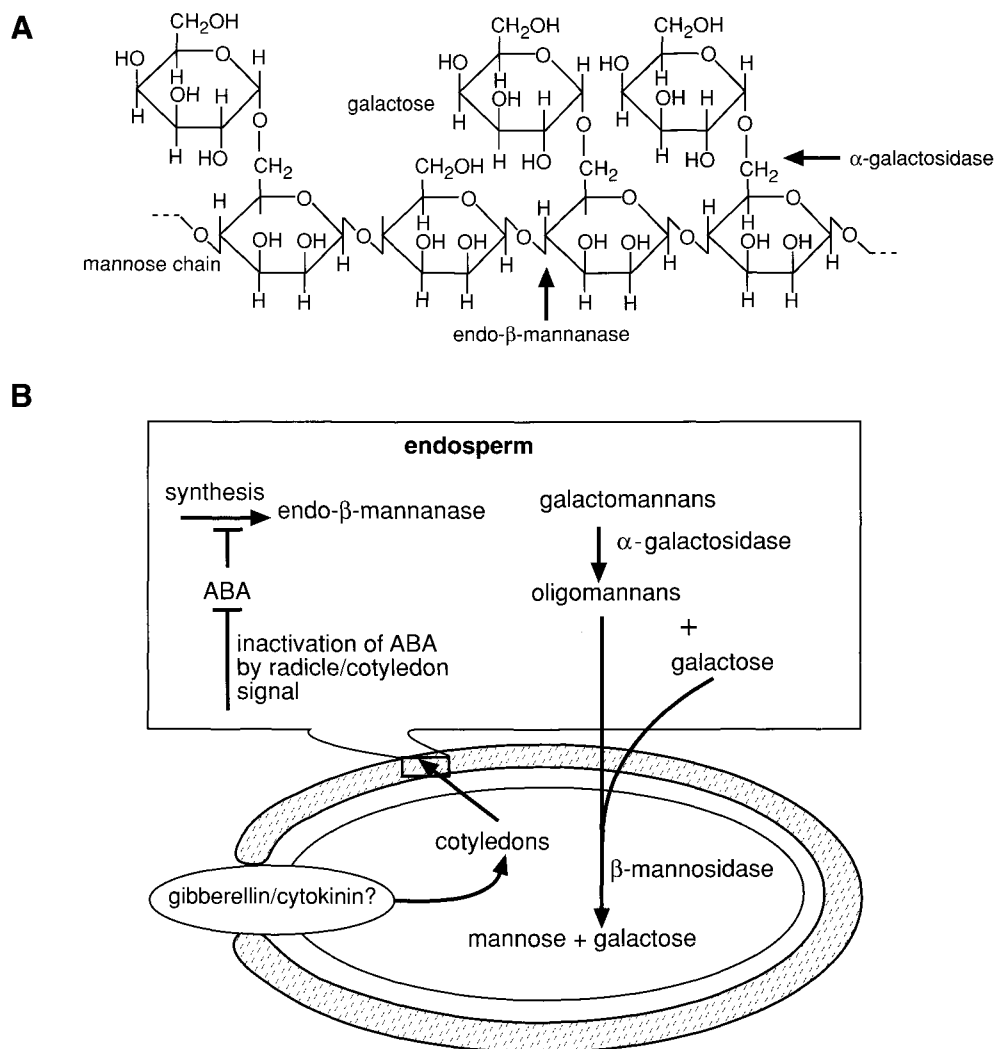


FIGURE 19-16 Hydrolysis of galactomannan-rich cell walls in lettuce endosperm. (A) Structure of galactomannans and the sites of cleavage by endo- β -mannanase and α -galactosidase. (B) Model for the hydrolysis of galactomannans in endosperm cell walls. During or immediately following germination (radicle is shown protruding out of the seed coat), a chemical signal (GA/CK?) is thought to move from the embryonic axis via cotyledons to the endosperm. The synthesis of endo- β -mannanase in the endosperm is kept repressed by an endogenous inhibitor (probably ABA). This repression is overcome in response to the signal from the radicle/cotyledon. α -Galactosidase catalyzes the removal of the galactoside side chains of galactomannans in the endosperm. Oligomannans released from the endosperm cell walls diffuse to the cotyledons where they are hydrolyzed to mannose by the action of β -mannosidase. Reprinted with permission from Bewley (1997).

some hormone from the embryonic axis moves to the cotyledons (or from the embryo to the endosperm or the megagametophyte) and triggers the activation or *de novo* synthesis of hydrolases. Despite much effort, however, no clear picture has emerged.

In many cases, a positive effect of GAs, auxins (IAA, 2,4-D), or cytokinins (kinetin, benzyladenine) on the mobilization of reserves in detached cotyledons or endosperm has been reported. Enzymes, such as malate synthase or α -amylase, have been reported to be induced and, in some cases, it has been suggested

that the hormone is produced in the embryo and migrates to the storage organ. However, in no case is the production of an enzyme as massive or as clear cut as that of α -amylase in the cereal aleurone layer. Moreover, contradictory reports show no clear correlation between hormone application and enzyme activity in detached organs.

The situation is complicated because the main storage organs, cotyledons or endosperm in most dicots and megagametophyte in conifers, are composed of living parenchyma cells, which may be capable of

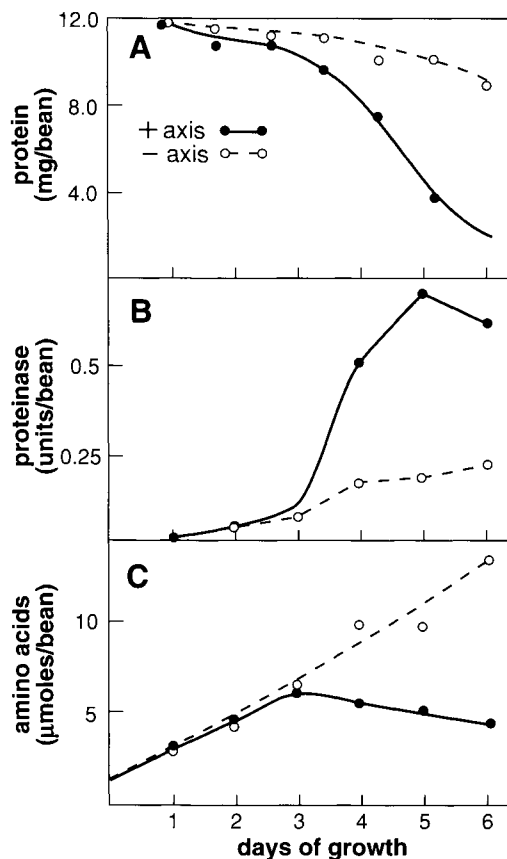


FIGURE 19-17 Role of the axis in protein mobilization in cotyledons of mung bean. (A) Changes in amounts of total reserve protein. (B) Extractable proteinase (total enzymes that could hydrolyze casein) activity. (C) Amounts of soluble amino acids in the cotyledons. Total period of incubation: 6 days. Solid line, intact seedlings; broken line, detached cotyledons. From Kern and Chrispeels (1978).

producing their own hormones. If they do, without a stimulus from the embryo, the effect of an exogenous hormone would not be very clear. With the availability of analytical techniques for the determination of picomole quantities of hormones such as IAA, GAs, and cytokinins, as well as cDNA clones for several GA synthesis enzymes (although not IAA and cytokinins), it should now be possible to determine whether detached cotyledons (or endosperm or megagametophyte) synthesize their own hormones. Similarly, the availability of cDNA clones for various hydrolytic enzymes should make it possible to determine whether any are induced in detached storage organs by exogenous hormones.

4. SECTION SUMMARY

Seedling growth, which starts when germination is completed, signals a shift in priorities to a rapid growth

of root and shoot and establishment of a photosynthetic seedling. In the process, reserve foods are hydrolyzed and provide fuel for energy and building blocks for new macromolecules. The hydrolysis of food reserves in cereal grains is regulated by gibberellins produced by the embryo and mediated *via* a battery of enzymes that are activated or synthesized *de novo* in the scutellum (an embryonic tissue) and aleurone tissue (part of the endosperm). Enzymes include those involved in lipid breakdown, membrane synthesis, hydrolysis of cell wall polysaccharides, various exo- and endoproteases, peptidases, and amylases, as well as nucleases. Some of these enzymes are used *in situ*, but others are secreted into the starchy endosperm. GAs seem to be involved in the maintenance of an active secretory machinery by elevating intracellular-free Ca^{2+} concentrations, which are required for the exocytosis of vesicles laden with enzymes. Many of the hydrolases produced have acidic pH optima, and GAs optimize their activities by promoting an acidification of the aleurone/endosperm environment. The hydrolysis of reserve foods in dicots and conifers during seedling growth is well substantiated and genes for many key enzymes have been cloned, but it is not clear whether the embryo or embryonic axis sends out a signal for the mobilization of reserves in the main storage organs, the endosperm or cotyledons in dicots or the megagametophyte in conifers. In some cases, the embryo has been shown to exercise a regulatory control, and GAs, as well as auxins and cytokinins, have been implicated in the induction of hydrolytic enzymes, but in no case is the response as clear cut or as massive as in cereal grains. The end products of hydrolysis of reserve foods, particularly sucrose and glucose, have, in many cases, been shown to exercise a negative feedback control over the expression of genes encoding some of the hydrolyzing enzymes.

SECTION III. SEED DORMANCY

1. THE PHENOMENON OF SEED DORMANCY

Seed dormancy is defined in a functional sense. Seeds that do not germinate given optimal conditions of temperature, water, and oxygen are considered **dormant**, whereas those that do germinate are considered **nondormant**. Because almost any seed will either germinate or go moldy and deteriorate given a long enough time, germination is measured over a defined incubation period (usually 14 or 21 days) under specified light/dark and temperature conditions.

Seed dormancy is widespread in nature and of extreme importance to the survival of natural populations, but it is still not well understood. As mentioned earlier, imbibed dormant seeds show very much the same changes in metabolism and respiration rate as imbibed nondormant seeds, but whereas the latter go on to complete germination by emergence of the radicle, dormant seeds never enter that phase. It is as if some switch fails to be turned on. The nature of the switch, despite much work, is unknown.

Dormancy is not an all-or-none phenomenon. Individual seeds in cross-breeding populations, or whole lines/families in self-breeding populations, differ quantitatively in the extent or degree of dormancy; some have none, others are highly dormant, and still others are in between. Thus, dormancy has a genetic basis, but its expression and degree are affected by developmental cues and environmental factors (see Chapter 18). In some cases, the same plant may produce nondormant seeds earlier in the season and highly dormant seeds later in the season; seeds of the same genotype growing in two different locations may show different degrees of dormancy. Such variability in dormancy allows seeds to stagger their germination in the soil (referred to as seed bank) and confers an adaptive advantage for survival of the species through varying environmental conditions.

2. TYPES OF SEED DORMANCY

Seed dormancy is divided into two basic types: **primary dormancy**, which is acquired during seed development/maturation, and **secondary dormancy**, which results from factors after the seed is shed. For instance, seeds that are hydrated and potentially ready to germinate may be exposed to supraoptimal temperature or salinity conditions and be inhibited from germination, thus becoming "secondarily" dormant. In some cases, secondary dormancy has been related to a rise in endogenous ABA content. This chapter deals only with primary dormancy.

Primary dormancy can be due to many reasons, which are difficult to classify. In some seeds, the seed coat or other structures surrounding the embryo prevent the embryo from completing germination. If these barriers are removed mechanically, the embryo is able to emerge. This type of dormancy is referred to as **coat-related** or **coat-enhanced dormancy** (the term "coat" is used in a nontechnical sense to include the testa, pericarp, remains of the endosperm, remains of the nucellus or perisperm, and, in conifers, the outer cell layer of megagametophyte). In other seeds, removal of

the coat has no effect, and dormancy is inherent in the embryo. This type of dormancy is referred to as **embryo-related dormancy**. Seeds with embryo-related dormancy require the perception of some environmental signal, usually chilling or exposure to light, before they will germinate. However, seeds with coat-related dormancy may also require environmental signals.

Some seeds fail to germinate because, when shed from the mother plant, they still have a very **immature** embryo. For example, seeds of ash (*Fraxinus excelsior*), cow-parsnip (*Heracleum sphodylium*), celery (*Apium graveolens*), ginseng (*Panax quinquefolius*), and most members of Orchidaceae have small, rudimentary embryos that require a period of growth and tissue and organ differentiation before they can germinate. This growth is at the expense of the stored reserves in the endosperm. In orchids, the seeds need to be exposed to a period of "maturation" in warm air (15–25°C), during which embryo development continues. They also must have mycorrhizae associated with them for development. In some other cases, the embryo displays dormancy as well. In ash, for instance, embryo growth occurs at normal temperatures, but the radicle protrudes only after exposure to a chilling temperature. In cow-parsnip, chilling seems to be required for the elaboration/release of some factor from the embryo that induces the degradation of protein reserves in the endosperm. In celery, light and GA seem to be necessary. In ginseng, the seeds require alternating regimes of chilling and warm temperature for many months before they will germinate (Fig. 19-18).

3. COAT-RELATED OR COAT-ENHANCED DORMANCY

In many species, the coat either acts as a barrier to permeation by water or gases or is mechanically too strong. For example, in cocklebur (*Xanthium*), the seed coat is reported to be impervious to gases. Waxy deposits occur on the surfaces of many seeds and can prevent water uptake. However, in yellow cedar (*Chamaecyparis nootkanensis*), a conifer, the barrier to water uptake is not the waxy deposit on the seed coat, but rather the cuticle on the surface of the megagametophyte (Fig. 19-19). In some other cases, the micropyle is plugged, and the plug has to be removed before water uptake can occur (e.g., *Melilotus alba*, *Albizia lophantha*). Some seeds have hard, lignified testa, which the embryo is unable to break through (e.g., *Mesquite*, a legume, and many nuts). In the field, seeds with mechanically strong or impervious coats

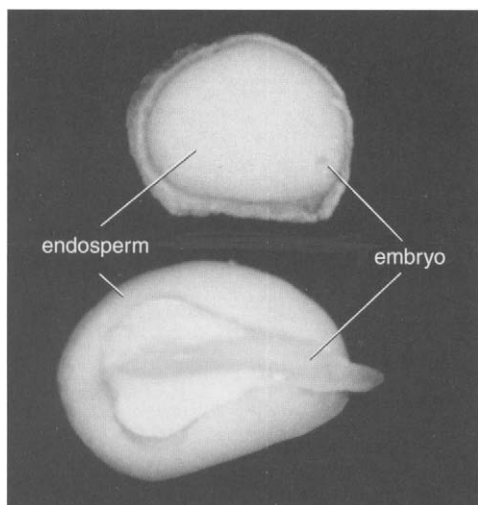


FIGURE 19-18 Seeds of ginseng (*Panax quinquefolius*) cut longitudinally to expose the embryo and endosperm. The top seed was shed from the parent plant late in August. The rudimentary embryo is about 0.5 mm long and the seed coat is not cracked. These seeds are placed in inground stratification boxes in late August. Seeding into growing beds takes place the following August when the embryo is about 2.5 mm long and some of the seed coats are cracked. By late October, some 14 months later, the embryo will have grown to about 4 to 5.0 mm long (the seed on bottom). Note the change in relative sizes of the embryo and endosperm. The seed then goes through another cold period and germinates the following May. Courtesy of John Proctor, University of Guelph.

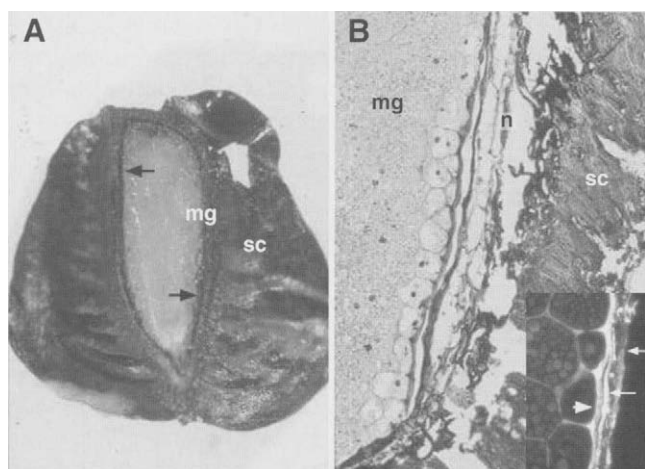


FIGURE 19-19 Seed of yellow cedar (*Chamaecyparis nootkanensis*). (A) Whole seed cut open longitudinally to show the seed coat (sc), megagametophyte (mg), and embryo (the radicle is seen more clearly toward the bottom end). Arrows point to the megagametophyte/nucellus boundary. The seed coat in yellow cedar has broad lateral wings. (B) Longitudinal section of the seed showing part of the megagametophyte, outermost cell layer of the megagametophyte with cuticle, remains of the nucellus (n), and inner part of the seed coat with sclerenchyma cells. Storage protein bodies occur in the cells of the megagametophyte. (Inset) Higher magnification of outermost cell walls of the megagametophyte (arrowhead), exine thinner, longer arrow, and cuticle (shorter arrow), which comprise the permeability barrier. From Tillman-Sutela and Kauppi (1998).

experience weathering action by dirt, sand, or water. For germination in the laboratory or greenhouse, these seeds are often subjected to scarification—physical abrasion or breaking of the seed coat, or even acid treatment for a short time (e.g., *Chenopodium rubrum*). In others, the remains of endosperm or perisperm, although not hard and lignified, have thick cell walls rich in hemicelluloses that the radicle is unable to penetrate. These seeds require weakening of the coat cell walls by specific enzymes (e.g., *Datura*, tomato, lettuce).

There are also some very interesting cases where the seed coat has to be exposed to “weathering action” in the digestive tract of animals before germination can occur. For example, seeds of some species of *Acacia* in Africa have to pass through the digestive tracts of elephants before they will germinate.

4. SOME SEEDS HAVE A REQUIREMENT FOR AFTERRIPENING

Freshly harvested seeds of many plants do not germinate or show poor uneven germination under optimal incubation conditions. They require warm, dry storage for varying periods (a few weeks to 8–12 months at 20–40°C during which seeds lose water) to break dormancy (Fig. 19-20). The phenomenon is known as **afterripening** and has been especially investigated in grasses (e.g., oat, wheat, rice, brome grass) and dicots (e.g., *Xanthium*, *Datura*, *Brassica*, *Arabidopsis*, tomato, and species of maple).

Individual lines or cultivars of species differ quantitatively in the duration of warm, dry storage required; some require none and are nondormant, whereas others require long periods and are highly dormant. Thus, in wheat the cv Brevor is highly dormant, whereas the cv Greer is nondormant. In wild oat (*Avena fatua*), some lines, e.g., MON73 and AN127, are highly dormant, whereas line CS40 is nondormant (Fig. 19-21). Cross-hybridizations among the three lines of wild oat reveal that at least three genes, but possibly many more, are involved in determining the requirement for afterripening. Genetic studies on dormancy are still very few.

Seeds that require afterripening may show coat-related dormancy, i.e., if the coat is removed, the embryo does not require afterripening (e.g., wheat, barley, oat, *Arabidopsis*, tomato). In other cases, there is true embryo-related dormancy; removal of the coat has no effect and the seeds still require afterripening [e.g., some lines of wild oat (*Avena fatua*) and brome grass (*Bromus secalinas*)].

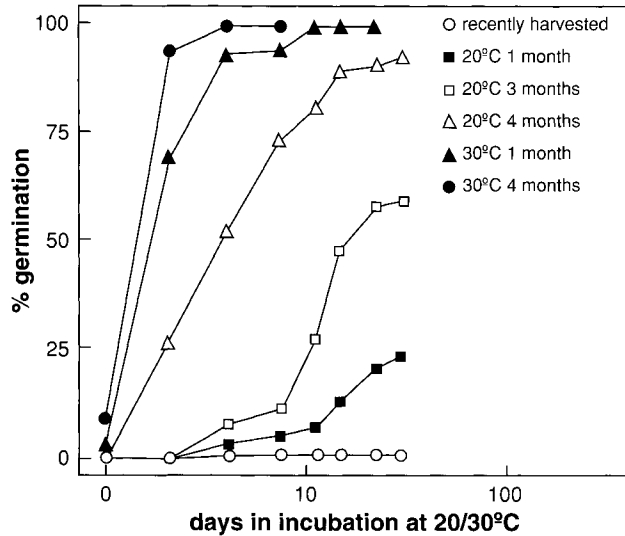


FIGURE 19-20 Germination of cheat grass (*Bromus tectorum*) seeds. Freshly harvested seeds and seeds stored dry at 20 or 30°C for 1 to 4 months were incubated for germination under identical conditions at night/day temperatures of 20/30°C. Freshly harvested seeds failed to germinate. Other curves show an inverse relationship between temperature and duration of dry storage and breaking of dormancy. Adapted from Allen *et al.* (1995).

4.1. Afterripening May Involve Changes in Endogenous GAs and ABA

The biochemical changes that occur in seeds during afterripening are unknown, although GAs and ABA are both implicated. In seeds of the wild oat line MON73, which show strong embryo-related dormancy, exogenously supplied gibberellin breaks the dormancy in a concentration-dependent manner (Fig. 19-22). The hybrid line, a cross between MON73 and a nondormant line SH430, shows an intermediate GA requirement. These data relate the GA requirement with the extent of dormancy of seeds and, by inference, with the requirement for afterripening. In other words, seeds proportionally more dormant or requiring longer afterripening treatment require a longer incubation period in GA or a higher concentration of GA in order to germinate.

In tomato, the cv Moneymaker, which shows coat-related dormancy, the dry storage of seeds is reported to lower the ABA content. Moreover, the afterripening requirement is bypassed by exogenously supplied gibberellin A_{4/7}. A similar effect of GA is reported for *Arabidopsis* seeds, which also show coat-related dormancy. Thus, it seems that in both coat-related and embryo-related dormancy gibberellin treatment can substitute for the afterripening requirement.

A correlation between gibberellin treatment and cold temperature is also known for many physiological

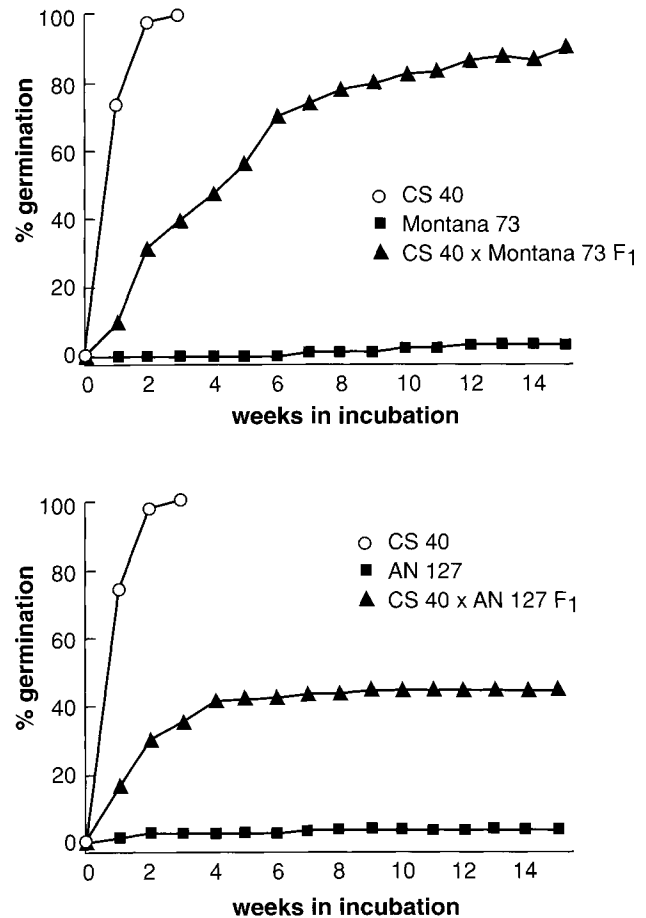


FIGURE 19-21 Seed dormancy in three lines isolated from populations of wild oat (*Avena fatua*). Line CS40 has little or no requirement for afterripening, and freshly harvested seeds germinate 98% within 2 weeks of incubation at 20°C. In contrast, seeds of Montana 73 (MON73) or AN127 are highly dormant and show no germination in a 15-week experimental period. F1 hybrids between CS40 and MON73 (top) or between CS40 and AN127 (bottom) show intermediate dormancy. From Jana *et al.* (1979).

processes. In this connection, it is interesting to note that dormant seeds of many species requiring afterripening (e.g., brome grass, wild oat), when freshly harvested, germinate better at lower (10/20°C) than at higher (20/30°C) night/day incubation temperatures. Also, some ecotypes of *Arabidopsis* germinate better if prechilled at 2–5°C for a few days.

4.2. Dormancy-Specific Proteins

Following an afterripening treatment, dormancy is broken, but seeds stay quiescent unless provided with the appropriate incubation conditions for germination. Comparisons of polypeptide profiles on two-dimensional gels of proteins extracted from dor-

mant but hydrated vs afterripened, but quiescent embryos of wheat and oat (also, of *in vitro*-translated proteins from mRNAs extracted from the same sources), reveal some differences, but have not yielded any polypeptides that could be said to be instrumental in imposing dormancy; i.e., they are present in dormant embryos but decline on breakage of dormancy.

Specific lines of wild oat, wheat, brome grass, and so on with strong embryo-related dormancy have also been used for the isolation of cDNA clones. Several clones specific to dormant embryos have been identified by differential screening or differential display. Transcripts for these clones are expressed in dormant dry embryos and increase severalfold on hydration, whereas their expression is low or absent in nondormant, germinating embryos. They can be induced by exogenous ABA in both dormant and nondormant embryos, an effect that is nullified by exogenous GA₃. Some of these clones have been found to be related to stress tolerance, they encode late embryo abundant (LEA)-type proteins, proteins associated with sugar metabolism, and antioxidants (see Chapter 18). Unfortunately, no cDNA clones have been identified so far that have a specific relationship to dormancy.

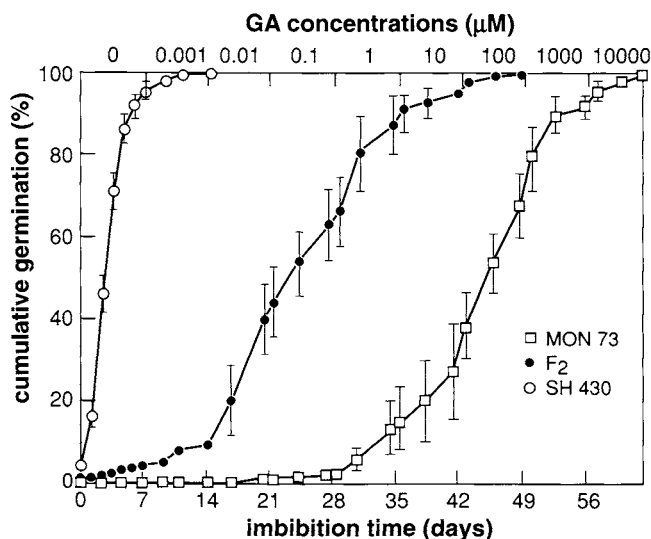


FIGURE 19-22 Substitution of an afterripening requirement by gibberellin A₃ in seeds of wild oat (*Avena fatua*). Seeds of MON73 (highly dormant), SH430 (nondormant), and the F₂ generation of MON73 × SH430, all without afterripening, were exposed to progressively higher GA₃ concentrations. No GA was added during days 1–7. On the eighth day, the concentration was increased to 1 nM and was increased by a factor of 10 each week thereafter (see top axis) until all seeds had germinated. Vertical bars represent the standard errors of the means. From Fennimore and Foley (1998).

5. SOME SEEDS HAVE A REQUIREMENT FOR CHILLING

Seeds of many plants require exposure to cold temperature (2–5°C) in an imbibed state to break their dormancy, a process known as **cold stratification**, or **chilling**. The dormancy itself may be coat related or embryo related. The chilling requirement is widespread in plants that are native to temperate regions with a cold winter and shed seeds in late summer early fall, e.g., many conifers, also hardwoods, such as oak (*Quercus*), ash (*Fraxinus*), sycamore (*Acer*), hazel nut (*Corylus*), and apple (*Malus*), as well as shrubs and bushes. The phenomenon also extends to many annual weeds (e.g., *Capsella*, *Chenopodium*, *Amaranthus*, *Sorghum*, some lettuce species). This requirement for chilling is an adaptive device to prevent germination at the onset of winter, when the young seedlings are likely to be killed by the first frost.

Different species have different requirements for the duration of the cold period, from a few days to several months. Usually within a species, the longer the chilling period, the more complete is germination (Fig. 19-23). Some species require alternating periods of warm and cold temperature (e.g., yellow cedar) whereas others require partial drying (reduction in moisture content) for part of the duration (e.g., *Abies* sp.). Different ecotypes of the same species also show differences in chilling requirements. Thus, the coastal variety of Douglas fir (*Pseudotsuga menziesii*) in Pacific northwest Canada and the United States has little or no requirement for chilling, whereas the interior variety has a specific requirement for about 3 weeks of chilling.

The protocols for chilling of seeds of various conifers and hardwoods of commercial importance have been worked out by trial and error, but the following question, remains unanswered. “What does the cold temperature do?” The question has a practical importance in that the answer may help expedite the germination of seeds, such as those of true firs and yellow cedar, which take months of stratification.

5.1. Hormonal Changes

An immense body of literature indicates a rise in gibberellin-like substances and a decline in inhibitors such as ABA following chilling treatments. Most of this work has been done using bioassays, and the identity of the gibberellins is unknown. Relatively few studies have used gas chromatography-mass spectroscopy for the identification of GAs (see Fig. 19-24) and ABA.

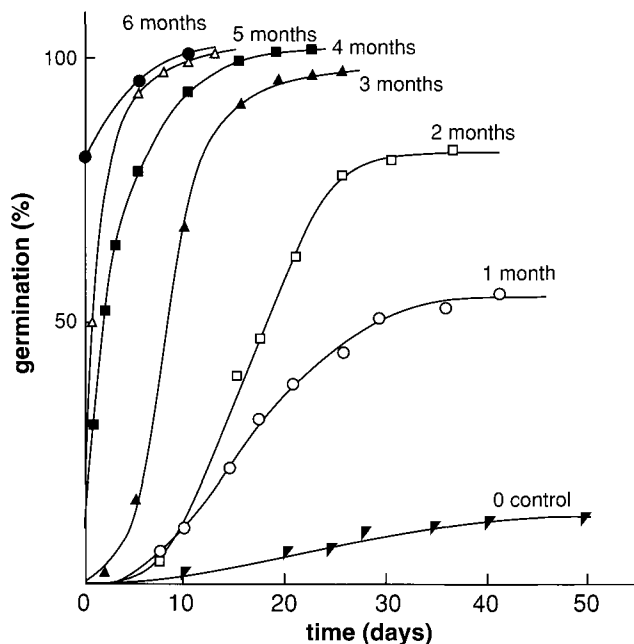


FIGURE 19-23 Effects of different periods of chilling at 5°C on subsequent germination of sugar pine (*Pinus lambertiana*) seeds at 20°C. From Baron (1978).

Application of GA₃ or a mixture of GA_{4/7} to dormant seeds to alleviate the chilling requirement has

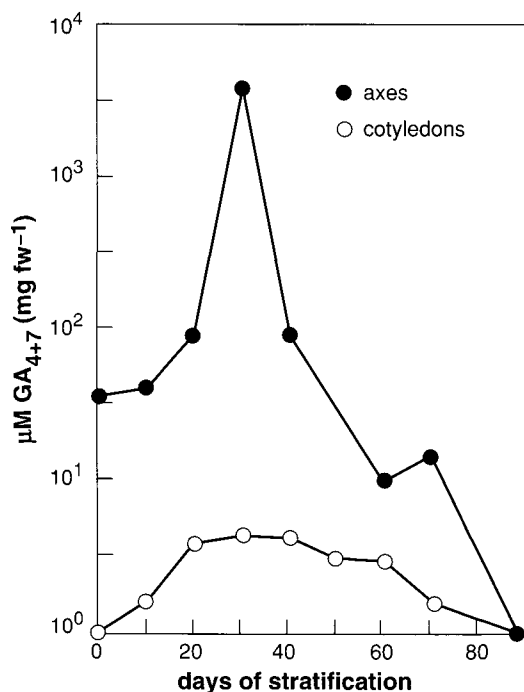


FIGURE 19-24 Changes in endogenous GAs following chilling of apple seeds. The concentration of GA_{4/7} shows a sharp rise between 20 and 40 days after the beginning of stratification in both the embryonic axis and the cotyledons. Reprinted with permission from Halinska *et al.* (1987), ©Munksgaard International Publishers Ltd., Copenhagen, Denmark.

given conflicting results: in some cases, it alleviates the cold requirement altogether, in others only partially, and in still others very little or not at all. Some data also suggest that ABA is leached from seeds by soaking at warm temperature (20–25°C), but dormancy is not broken and the seeds do not germinate unless given a cold treatment.

The complex interaction of various factors that affect dormancy is illustrated by the seeds of a conifer, yellow cedar (*C. nootkanensis*). These seeds show dormancy that is primarily, but not exclusively, coat imposed. Leachable inhibitors, such as ABA, are present in the embryo, as well as megagametophyte, and play a role in maintaining the embryo in a nongerminative state. In addition, an impervious barrier at the megagametophyte/nucellus boundary (see Fig. 19-19) also plays a role in preventing radicle protrusion. Studies show that chilling, gibberellins, ABA content, and sensitivity of embryos to ABA all play a role. For instance, intact seeds require a period of about 4 weeks of warm soaking followed by 8 weeks of chilling to give good germination (~85%). During this period, ABA levels decline in the embryo to about half the level before the treatment, but they do not decline in the megagametophyte. So, in intact seeds, ABA may still inhibit germination. However, in the same period the sensitivity of excised embryos to inhibition by ABA declines by about 10-fold (Fig. 19-25). Seeds supplied with a mixture of GA_{4/7} at room temperature show only a marginal improvement in germination, but if GA treatment is combined with a warm soak and chilling, the stratification period can be reduced considerably. This improvement by GA in combination with chilling may work in concert with the decline in sensitivity of embryos to ABA to enhance germination in stratified seeds. Finally, the physical barrier to radicle protrusion, the outer boundary of the megagametophyte, may be weakened by enzymes that are synthesized or activated by low temperature or GA.

5.2. Changes in Proteins

There have been some studies on protein profiles in dormant embryos vs chilled, nondormant embryos, but no specific proteins or mRNAs have been identified. In a study on *Acer saccharum*, which has a strong chilling requirement, seeds were kept under two temperature regimes, soaked at 15°C, which is nonpermissive, and soaked at 4°C, which is permissive for breaking dormancy. Several polypeptides were identified that were present in dormant embryos, but whose incidence was markedly lowered in seeds given the cold treatment. The identity of the proteins and their specific function are unknown.

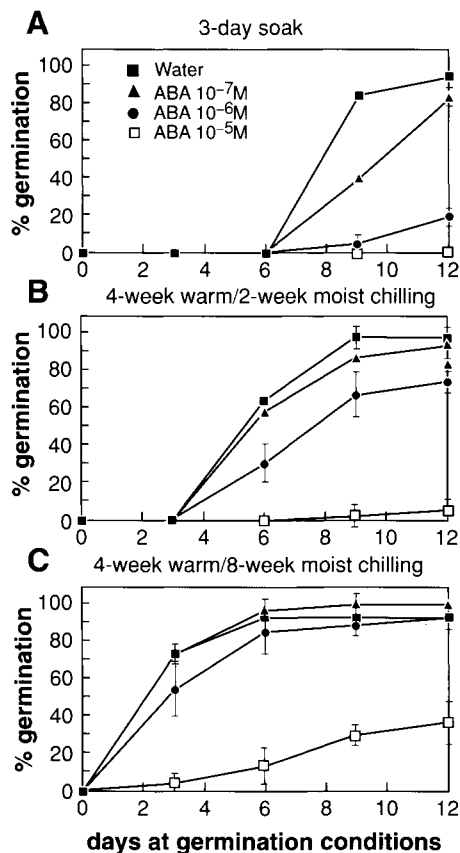


FIGURE 19-25 Sensitivity of excised embryos of yellow cedar (*Chamaecyparis nootkanensis*) to inhibition of germination by ABA. Seeds were given various dormancy-breaking treatments. Subsequently, embryos were excised and tested for germination under three ABA concentrations (10^{-7} , 10^{-6} , and 10^{-5} M). Data for excised embryos exposed to 10^{-6} M ABA show that 50% germination occurs after 3 days of incubation if the seeds are given a 4-week warm and 8-week moist chilling, and after 8 days of incubation if the seeds are given a 4-week warm and 2-week moist chilling; the embryos require much longer than 12 days of incubation if the seeds are given only a 3-day soak. Results from embryos exposed to 10^{-7} or 10^{-5} M ABA are similar. Thus, the protocol of warm, moist chilling reduces the sensitivity of excised embryos to inhibition of germination by ABA. Data based on three replicates of 10 embryos each. Germination percentages for whole seeds under the same treatments were A, $0 \pm 0\%$; B, $6.7 \pm 1.7\%$; and C, $85 \pm 2.9\%$. From Schmitz *et al.* (2000).

6. SOME SEEDS REQUIRE EXPOSURE TO LIGHT

Seeds that have a specific requirement for light for germination are referred to as **photoblastic** seeds. The phenomenon is common in herbaceous and pioneer species, which produce large numbers of small seeds with relatively little food reserves. Many of these photoblastic seeds require a brief exposure to red light (660 nm) while in a wet state. The photoreceptor in this case is phytochrome, and the response has been

well investigated in seeds of *Datura ferox* (a tropical weed) and lettuce (*Lactuca sativa*). Indeed, phytochrome was discovered after some remarkable observations on red/far-red reversal of lettuce seed germination (see Chapter 26). Light-requiring seeds also show sensitivity to cold temperature, and gibberellins and, in some cases, the light requirement can be bypassed by the exposure of seeds to chilling ($2\text{--}5^{\circ}\text{C}$) or to GA. Both in lettuce and *Arabidopsis* seeds, it has been shown that red light induces the transcription of genes encoding specific isoforms of 3β -hydroxylase, the enzyme that catalyzes the conversion of GA_{20} to the biologically active GA_1 . Thus, the roles of phytochrome and gibberellin in the germination of red light-requiring seeds have been partially clarified.

Many photoblastic seeds (e.g., *D. ferox*, tomato, lettuce, *Arabidopsis*) also show coat-enhanced dormancy, which is related to the presence of an endosperm with galactomannan-rich cell walls. In lettuce, for example, it has been thought for a long time that phytochrome action leads to the production of gibberellins in the embryo, which migrate to the endosperm where they bring about the synthesis of endo- β -mannanase and α -galactosidase (see Fig. 19-16). While phytochrome action has been implicated in the synthesis of active GAs (see Chapter 26), no direct relationship has been established between phytochrome action and synthesis of wall-hydrolyzing enzymes in lettuce. With the cloning of the gene for an isoform of endo- β -mannanase in tomato, which is induced by GA, it may be possible to establish connections among phytochrome action, gibberellins, hydrolysis of cell walls in the endosperm, and radicle emergence.

7. SECTION SUMMARY

Seed dormancy is a phenomenon of considerable importance in the survival of plants. As outlined in Chapter 18, ABA, present in sufficient concentration at certain stages in seed development, is the inducing factor for seed dormancy, but the depth (or extent) of dormancy induction depends on the genetic makeup of the plant and on environmental factors, such as prevailing temperature, relative humidity, and day length. The variety of factors that influence seed dormancy makes it a highly complex phenomenon to study. However, the resultant variability in the dormancy of seeds of a species is extremely beneficial to its survival because it enables the seeds in the seed bank to stagger their germination in the field. Primary dormancy, which is induced during seed development, is of several types. Coat-related dormancy is due to the tissues that surround the embryo acting as

a mechanical or permeability barrier. Embryo-related dormancy resides in the embryo itself and, for breakage, requires warm, dry storage, chilling, or perception of red light *via* a phytochrome. No proteins or cDNA clones specific to embryo-related dormancy have yet been identified either for seeds requiring afterripening or for those requiring chilling. Gibberellins are involved because in many cases, seeds, which require afterripening or chilling, germinate if supplied with GA in the absence of those treatments. Many seeds requiring an exposure to red light show coat-enhanced dormancy. They germinate in the presence of GA without red light treatment, and it has been shown that an exposure of seeds to red light causes the synthesis of biologically active GA. This GA may then induce the synthesis of and/or activate the enzymes that hydrolyze the cell walls that cause dormancy. Expression of a GA-induced endo- β -mannanase gene in tomato seeds supports this hypothesis. Chilling of seeds also bypasses the red light requirement, but how chilling and gibberellins interact is not clear. Gibberellins are probably only one factor in the breakage of seed dormancy. An interaction with endogenous ABA or sensitivity to ABA may also be involved.

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20

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1. INTRODUCTION

Plants accumulate reserve foods (proteins, lipids, starch) in their seeds, which are hydrolyzed after germination and utilized for the heterotrophic growth of the seedling (see Chapters 18 and 19). Plants also store reserve foods in their vegetative parts, such as stems, roots, leaves, and floral axes. Such storage in perennial trees and shrubs, growing in temperate regions, has been known for a long time. In these plants, large amounts of food reserves are stored in xylem and phloem parenchyma cells in stems and roots in late summer/early fall; reserves that are hydrolyzed the following spring to provide sugars and amino acids for new growth. The types of reserves stored vary with species. For instance, conifers store large amounts of proteins and lipids, but not much starch. Sugar maple (*Acer saccharum*) stores starch—maple syrup comes from the hydrolysis of starch stored in xylem parenchyma cells. Some perennials are difficult to eradicate in gardens because of the vast reserves stored in their roots (e.g., blackberry). If the aerial parts are cut or destroyed, new shoots keep coming forth until the underground reserves are depleted. Less well known is the phenomenon that many herbaceous annuals, such as soybean, bean, and *Arabidopsis*, also accumulate reserves in their vegetative tissues. These reserves are often accumulated in leaves and stems and are hydrolyzed at the time of flowering to support seed development. The subject of reserve foods in vegetative organs is vast, but surprisingly little is known about the physiological and environmental regulation of such storage. The first section of this chapter deals with a small segment, that concerned with the storage of proteins in vegetative organs of annuals and perennials. Section II deals with another mode of storage: the production of special organs for storage namely, with tuber formation in potato.

Plants, like all organisms, senesce and die. Because plants have an open form of growth, the aging or senescence often does not extend to the whole plant. In perennials, for instance, leaves and flowers senesce and die, but the rest of the plant continues to live for many years. Even in annuals, leaves produced early in the life of the plant senesce and die while new leaves are still being produced. Senesced organs and, in many cases, perfectly healthy organs are discarded by a process of abscission. In the drama of plant life, senescence and abscission of organs are the "last exit." These two topics are covered in Sections III and IV of this chapter, respectively.

SECTION I. VEGETATIVE STORAGE PROTEINS (VSPs)

1. VSPs ARE A HETEROGENEOUS GROUP

Vegetative storage proteins are a heterogeneous group of proteins with seemingly diverse functions. What distinguishes a VSP from hundreds of other proteins in the cell is not always clear. Some proteins clearly fall into the category of storage protein. They are synthesized in times of plenty, stored, and hydrolyzed in times of need to provide building blocks for synthetic activities elsewhere. However, most VSPs have other known or presumed functions. Many VSPs are enzymes, such as acid phosphatases, lipoxigenases, and acyl hydrolases, but whether they function as enzymes is not known. Some lectins, ribosome-inactivating proteins (RIPs), and proteinase inhibitors (PINs) are stored in large quantities in vegetative tissues; they have a presumed function in plant defense. Ribulose biphosphate carboxylase/oxygenase (RUBISCO) is the most abundant plant protein. It is known to be degraded in leaves, and the resulting amino acids are mobilized for export to developing seeds, but it is not considered a VSP because it has a defined metabolic function and is not synthesized simply to be sequestered until utilized at a later time. This section first considers VSPs in herbaceous annuals, then storage proteins in perennial trees, and finally stored proteins with defense-related functions.

2. VSPs IN HERBACEOUS ANNUALS

2.1. Soybean

The occurrence of VSPs was first reported in the early 1980s in soybean (*Glycine max*) leaves. It was noted that leaves of this plant deposited large amounts of storage proteins whose incidence declined sharply at the time of seed growth. Removal of fruit pods (depodding), while not delaying leaf senescence, nonetheless arrested the decline in storage proteins and even restored the amounts to previous levels. It was later shown that VSPs accumulated in young growing leaves and stems as well. Since then, VSPs have been reported in various other plants, both annuals and perennials, and are present not only in leaves and petioles, but also bark tissue in stems and in vegetative parts of reproductive tissues, such as floral axes, ovary walls, and fruit pericarp. VSPs found in

different plant species can be very different and are distinct from the storage proteins found in seeds.

Several VSPs have been described in soybean. Two very similar proteins, VSP α and VSP β (M_r values of 27 and 29 kDa, respectively), are better characterized than others. Both are acid phosphatases, both are glycosylated, and both occur as homo- or heterodimers (proteins with two subunits that are either identical or dissimilar). Antibodies against these proteins have been prepared, and their genes cloned. The encoded proteins show ~80% sequence identity, and their transcripts are expressed in various tissues, with minor differences. They are expressed in abundance in young seedlings and in leaves, especially young growing leaves, and in pericarp of immature fruits (pods). The transcript abundance declines as tissues become older and more mature. Notably, transcripts accumulate even in tissues of seedlings that have been germinated and grown in dark, which means that the proteins can be synthesized from mobilized seed reserves. Antibodies raised against VSP α/β have been used to immunolocalize these proteins in such seedlings.

In leaves, proteins are stored in parenchyma cells adjacent to, or associated with, vascular tissues referred to as paraveinal mesophyll (Fig. 20-1). They are absent in palisade parenchyma, which is specialized for photosynthesis. A similar distribution in cells associated with vascular tissues is seen in other organs as well.

Because these proteins and their transcripts accumulate in young growing tissues, it appears that they are synthesized if the supply of nutrients, especially nitrogen, exceeds the demands for growth. The decline in protein amounts, coincident with seed development, suggests that they are hydrolyzed to provide building blocks for the synthesis of seed food reserves.

A 94-kDa protein in soybean, which is a lipoxygenase (LOX, see Chapter 12), shows a distribution pattern similar to that of VSP α and VSP β , but its expression and degradation patterns are different. Its mRNA is more highly expressed in older, mature leaves than in young leaves, and it does not seem to have a specific role in providing building blocks for the synthesis of seed proteins. The protein is maintained in pod-bearing plants longer than VSP α and VSP β after flowering and, on depodding, its levels actually decline (see Section 2.4).

2.2. *Arabidopsis*

Several genes encoding VSPs have been cloned in *Arabidopsis*. One homologue of soybean VSP α/β was reported with similar tissue distribution. More recently, two other genes, *AtVSP1* and *AtVSP2*, have

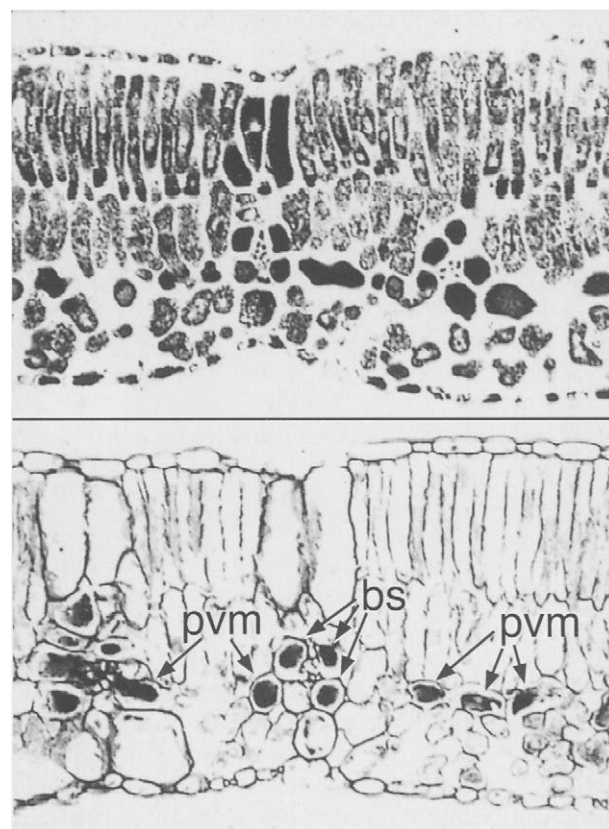


FIGURE 20-1 Vegetative storage proteins in leaf of soybean (*Glycine max*). (Top) Leaf cross section stained to show total protein distribution. Note that the paraveinal mesophyll (pvm) and bundle sheath cells (bs), as well as other mesophyll cells, all show staining for proteins. (Bottom) Section from the same leaf stained by an immunoperoxidase method for the localization of vegetative storage proteins, VSP α and VSP β . VSPs are localized to the paraveinal mesophyll and associated bundle sheath cells and are absent from other mesophyll cells. From Franceschi *et al.* (1983).

been cloned. They also encode proteins that are expressed similarly to soybean VSP α/β , as determined by promoter::GUS constructs and their transgenic expression in *Arabidopsis*. *AtVSP1* is strongly expressed in ovary tissues and in developing fruit walls, whereas *AtVsp2* is expressed predominantly in vegetative shoots, petioles, peduncles, and receptacles of floral organs.

2.3. Synthesis and Intracellular Location of VSPs

VSPs are immunologically distinct from seed storage proteins. They are encoded by their own genes, which are usually not expressed in seed tissues, just as genes for seed storage proteins are normally not expressed in vegetative tissues (although they can be expressed ectopically in vegetative parts).

Comparisons of deduced amino acid sequences of VSPs and seed reserve proteins from the same plant, such as soybean or *Arabidopsis*, do not show any significant homologies.

The intracellular processing of VSPs is not as well characterized as that for reserve proteins in dicot seeds. It is known, however, that VSPs in soybean or *Arabidopsis* do not assemble into hexameric or trimeric oligomers typical of legumins or vicilins (see Appendix 3). However, like seed storage proteins, some are glycosylated in the endoplasmic reticulum and are then stored in the vacuole, which becomes a protein storage vacuole. Targeting to the vacuole is confirmed by immunohistochemical staining using antibodies.

2.4. Inducing Agents

VSPs are accumulated under a variety of inducing stimuli. The most important signal seems to be the nutrient status of the plant or tissue. An abundant nitrogen and sugar supply promotes the accumulation of VSPs, as shown by cells in culture, or detached leaves, supplied with excess sugar or amino acids. VSP accumulation can also be enhanced by blocking the export of photoassimilates from a fully expanded leaf by inhibiting phloem transport through heat girdling of the petiole. Because VSPs in leaves of soybean (*G. max*) or bean (*Phaseolus vulgaris*) are mobilized to provide building blocks for syntheses of reserve proteins in seed tissues during "seed filling," removal of immature fruits results in an increased accumulation of VSPs in leaves (Table 20-1). Similarly, removal of shoot or root tips, which are actively growing and require nutrients, causes VSP accumulation.

Among hormones, jasmonates are very potent inducers of VSPs in soybean and *Arabidopsis*. Leaf explants or suspension cell cultures from soybean, even

whole plants of soybean exposed to MeJA, accumulate VSPs (Fig. 20-2). Such accumulation occurs even in nitrogen (N)-deficient plants, although the effect is more pronounced if the nitrogen supply is abundant. The proof that jasmonates are involved in VSP accumulation comes from certain mutants of *Arabidopsis* (e.g., *jar1*, for jasmonic acid-resistant, and *coi1*, for coronatine-insensitive) that are unable to respond to jasmonates (see Chapter 24 for a further discussion of these mutants). These mutants show poor induction of VSPs on wounding or exposure to MeJA. Although abscisic acid (ABA) shares many responses with jasmonates (see Chapter 12), neither ABA nor any other hormone induces these proteins.

Water deficit or wounding of plant tissue also induces VSPs in soybean, although it is possible that such induction occurs via the stress-induced synthesis of jasmonates (see Chapter 12).

2.5. Other Herbaceous Plants

Immunologically similar proteins that cross react with antisera raised against soybean VSP α/β have been found in many crop plants (e.g., bean, pea, alfalfa, tobacco, maize). These proteins also accumulate under high nitrogen. A tomato VSP, which is about 40% identical to soybean VSP α/β and which is an acid phosphatase, has been reported. A cDNA encoding a 28-kDa VSP in bean (*P. vulgaris*) has been cloned. It is strongly expressed in immature fruit pods and is induced by wounding. Both the amounts of the protein and its mRNA decline with senescence.

3. STORAGE PROTEINS IN DECIDUOUS TREES

Storage proteins are accumulated in stems and roots of many perennials (e.g., apple, maple, willow, poplar) in late summer/early fall and serve as a nitrogen source for early growth next spring. In deciduous plants, at least some of these reserves are synthesized from the products of the mobilization of nitrogen and carbon from senescing leaves before the latter are abscised (see Section III). These proteins, often described as "bark proteins," are stored in the vacuoles of parenchyma cells of the inner bark (i.e., cortex and phloem), the cambial zone, and the xylem parenchyma. The proteins are absent from the vacuoles of these same cells in summer (Fig. 20-3).

Several different types of bark storage proteins have been described. For instance, the main proteins in sugar maple (*Acer saccharum*) that are present in winter

TABLE 20-1 Amount of VSP in Soybean Leaves at Various Times after Flowering as Determined by Immunoassay and Densitometer Scans of Coomassie Blue-Stained Gels^a

Time after flowering (weeks)	\pm pods	Glycoproteins (% soluble)	
		Immunoassay	Gel scan
0	+	15	6
1	+	15	6
3	+	7	3
3	—	29	22
5	+	1	1
5	—	47	45

^aFrom Wittenbach (1983).

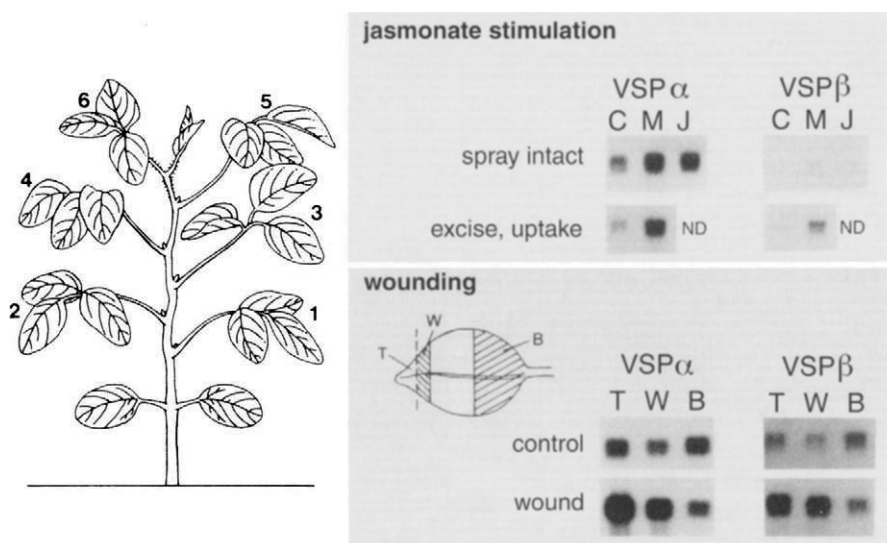


FIGURE 20-2 Effect of methyl jasmonate treatment or wounding on levels of VSP α and VSP β mRNAs in soybean (*Glycine max*) plants. A schematic drawing of a soybean plant is shown on the left. Trifoliolate leaves are numbered. (Right, top) Effect of methyl jasmonate and jasmonic acid. Plants were sprayed individually at the end of the day with 0.05% Tween 20 (control, C) or Tween 20 with 30 μ M MeJA (M) or Tween 20 with 30 μ M jasmonic acid (J); after 12 h in the dark, the third trifoliolate leaf was harvested. Alternatively, the third trifoliolate was excised under water from a single plant, and individual leaflets were incubated in the dark with their cut ends in water (C) or 10 μ M MeJA (M). Note that MeJA or jasmonic acid sprayed on intact leaves caused an enhanced accumulation of VSP α mRNA over control. Also, MeJA supplied via the cut petiole enhanced the transcripts of both VSP α and VSP β over the control. (Right bottom) Effect of wounding. The third trifoliolate leaf was wounded by cutting off the terminal 1 cm of the middle leaflet. After 12 h in the dark, samples were taken from the terminal segment (T), wounded segment (W), and the basal segment (B). Wounding enhanced the expression of VSP α and VSP β mRNA in the terminal and wounded segments. In each treatment, an identical amount of RNA was loaded per lane and hybridized with gene-specific probes. Adapted from Mason and Mullet (1990).

and are lost in summer are two proteins of 16 and 24 kDa. In willow (*Salix* sp.) and poplar (*Populus deltoides*), the main storage protein is about 32 kDa; it is immunologically similar in the two species and comprises roughly 30% of the total extractable protein from the bark tissue.

3.1. Are Bark Storage Proteins VSPs?

Bark storage proteins in perennials are formed under different environmental conditions than VSPs in herbaceous annuals. They are also utilized differently. The latter are induced by the nutrient status (i.e., overproduction of nutrients or removal of sinks), wounding, water deficit, and/or exposure to jasmonates and are utilized for the most part by hydrolysis and recycling of nutrients for seed development. In contrast, bark storage proteins are accumulated and hydrolyzed on a yearly cycle. In temperate climates, they are formed under shortening days and cooler temperatures of late summer/early fall. They are hydrolyzed in spring to provide nutrients for early growth before the full photosynthetic capacity is re-

stored. However, these differences between VSPs and bark storage proteins may be superficial.

For instance, in poplar, the 32-kDa protein is induced by short days, but it can also be induced under long days if the trees are exposed to a lower temperature, supplied with nitrogen, or if the sink demand is changed by stem girdling. Also, in poplar, wounding induces the transcription of a family of *Win* (for wound-inducible) genes, which are expressed both locally and systemically in leaves distant from the wounded leaf, provided they are connected by vascular tissues. One of the wound-inducible proteins, WIN4, is related to the 32-kDa bark storage protein. However, like soybean VSP α and VSP β , the WIN4 protein and transcripts are most abundant in young tissues, the shoot apex, and the youngest leaves. The protein is immunolocalized to the cells surrounding the vascular tissues. The concentration of the WIN4 protein and transcripts in leaf cells increase with nitrogen fertilization, wounding, and after treatment with MeJA in a manner similar to soybean VSPs. Thus, the WIN4 protein provides a link between the VSPs as known from soybean and the major bark storage

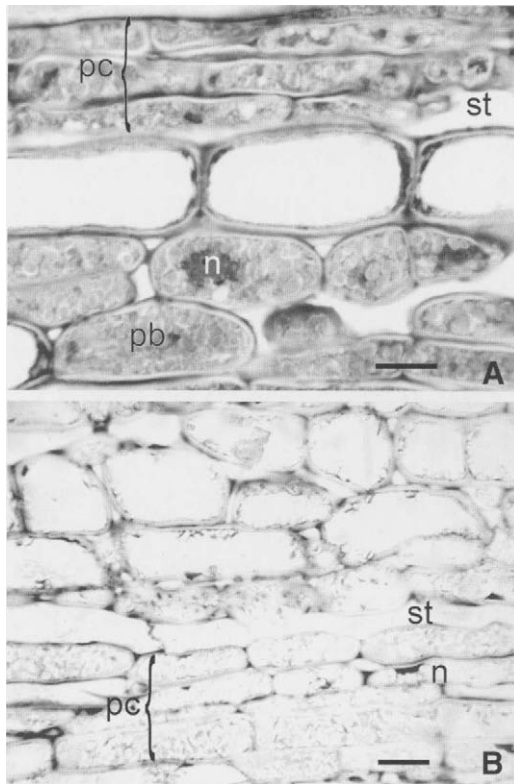


FIGURE 20-3 Comparison of winter (A) and summer (B) samples from bark of poplar (*Populus deltoides*). Protein bodies, stained positively with amido black, fill the phloem parenchyma cells in the winter sample. They are completely absent from the summer sample. Refractile cellular inclusions in the summer sample are starch granules. n, nucleus; pb, protein body; pc, parenchyma cell; st, sieve tube. Bar: 10 μ m. From Wetzal *et al.* (1989).

protein in poplar. The deduced amino acid sequence of the WIN4 protein has little similarity to soybean VSP α or VSP β . It also has no known enzymatic function.

In summary, storage proteins in deciduous trees and typical VSPs in herbaceous annuals represent a range of different types of proteins that are stored in parenchyma cells and accumulated under conditions of high N availability. The differences between them seem to be related to different lifestyles of annuals vs perennials. In the following section, another class of proteins, associated with plant defense, that can be stored in abundance, is considered.

4. DEFENSE-RELATED PROTEINS

Many other proteins are stored in vegetative tissues and are listed among VSPs. Three types of proteins with a role in plant defense are described below.

Patatin is the most abundant storage protein of potato tubers, comprising about 20–40% of total soluble protein. It occurs only in the tuber, not in shoots. It is an acyl hydrolase that catalyzes the nonspecific hydrolysis of a wide variety of acyl lipids, phospholipids, glycolipids, sulfolipids, and mono- and diacylglycerols. Unlike true lipases, it does not hydrolyze triacylglycerols (TAGs) and is active on lipids in solution rather than at the lipid/water interface. Patatin is sequestered in the vacuole; it is apparently inactive against the vacuolar membrane. If released by grinding, however, it can potentially hydrolyze the entire lipid content of the tuber within minutes. Patatin occurs in multiple isoforms, and its synthesis is inhibited by biologically active gibberellins (GAs). The role of patatin is unclear, although it is believed to serve a defensive role against insect herbivores because of its potent lipolytic activity.

Lectins are abundant in seeds of many plants, especially legumes, but they are also accumulated in vegetative tissues, such as leaves (e.g., soybean) or the bark of trees (e.g., *Robinia pseudoacacia*, *Sophora japonica*). Lectins are glycoproteins that recognize specific sugar residues, such as Glc, Man, Gal, and their combinations, and thus bind to oligosaccharide fragments. Some lectins consist of a single polypeptide with a single carbohydrate-binding domain; they serve as recognition molecules only. Others consist of one or more than one polypeptide, but have multiple carbohydrate-binding domains and, hence, are able to agglutinate cells or precipitate glycoconjugates. Several hundred lectins have been described in plants, the majority belong to the agglutinating type.

Lectins serve as potent defense chemicals against pathogens and herbivores. Substrate oligosaccharides for lectins are rare in plants, but occur commonly in bacterial or fungal cell walls and in the digestive tracts of insect and mammalian herbivores. Lectins bind to cell walls of pathogens and prevent cell multiplication or growth; they also bind to the epithelial lining of the intestinal tract of herbivores and interfere with digestion. Like other proteins involved in a defense against herbivory (e.g., α -amylase inhibitors, proteinase inhibitors) or pathogenic bacteria or fungi (e.g., chitinases, glycanases), lectins are stable under a wide pH range or heat. Many are also resistant to a variety of animal and insect proteases, although some animals have gut proteases that break down lectins.

Many dicots and monocots accumulate what are known as ribosome-inactivating proteins. RIPs are a special type of lectins; they bind to carbohydrates, but also act as N-glycosidases, which specifically remove adenine residues from RNA and DNA. Thus, they are very potent defense chemicals (e.g., ricin D in castor

bean). They occur not only in seeds, but also in vegetative organs. For example, in bulbs of *Iris hollandica* var Professor Blaauw (Iridaceae), a RIP is the most abundant protein.

5. SECTION SUMMARY

Vegetative storage proteins are a heterogeneous group of proteins that are accumulated in vegetative tissues, usually in vacuoles of parenchyma cells associated with vascular tissues. They are accumulated under high N or sugar conditions and are hydrolyzed to provide building blocks for synthetic activities elsewhere. Several VSPs from herbaceous plants, such as soybean, bean, and *Arabidopsis*, and from tree species, such as poplar, have been described. Many VSPs are enzymes, but their major function seems to be to serve as a nitrogen source to sink areas, including developing seeds and, in perennials, new growth in the spring. Inducing agents for VSPs in soybean include wounding, exposure of tissue to methyl jasmonate, or removal of sink tissues. Many defense-related proteins are accumulated in abundance, which permits degradation and recycling of nitrogen and carbon skeletons.

SECTION II. TUBERIZATION

1. TUBERS AND BULBS AS STORAGE ORGANS

Many plants, such as potato (*Solanum tuberosum*), yam (*Dioscorea rotundata*), and Jerusalem artichoke (*Helianthus tuberosus*), produce underground stem modifications known as tubers, which are used to store reserve foods. Other plants, such as sweet potato (*Ipomoea batatas*) and beet (*Beta vulgaris*), produce roots that are modified to store food reserves. Many other plants form bulbs, underground shoot apices surrounded by fleshy leaves that store food reserves (e.g., *Allium* sp., *Iris* sp., *Tulipa* sp.).

The potato is the world's fourth largest crop after the three cereals: wheat, rice, and maize. Moreover, among tuber- or bulb-bearing plants, it is the one best studied. This section deals with tuberization in the potato. For other tuber, root, or bulb crops, the reader is referred to the excellent book by Simpson and Ogorzaly (1995). The morphology and formation of the tuber are covered first, followed by the environmental and hormonal factors that regulate the tuberization process. Research on tuberization in the potato

is hampered partly by the fact that the cultivated potato (*S. tuberosum* ssp. *tuberosum*) is a tetraploid, which does not lend itself easily to mutagenesis and genetic manipulations, as is possible in *Arabidopsis*, tomato, or maize.

2. TUBERIZATION PROCESS

A potato plant produces underground shoots known as stolons, which grow horizontally (diageotropic growth). The stolons have nodes and internodes, but nodes produce only tiny scale-like leaves, which, like the rest of the stolon, do not become green. Tuberization begins by cell division and radial expansion of cells in the subapical part of the stolon, while growth at the apex ceases (Fig. 20-4). Continued divisions and radial expansion of cells lead to swelling and eventual shape of the tuber. Deposition of starch begins very early. The "eyes" in a tuber are the nodes—they may have buds; internodes are the intervening parts. The scale-like leaves are usually shed during harvest and handling. The dramatic change in morphology, from an elongating stolon to a radially expanding and swollen tuber, is accomplished by a changed pattern of growth of the constituent cells and is reflected in the arrangement of their cortical microtubules (MTs). Cells in the stolon show MTs arranged, as expected, mostly at right angles to the direction of cell elongation. In

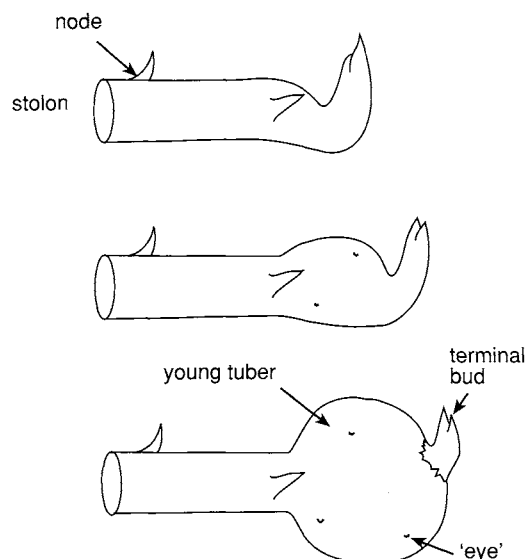


FIGURE 20-4 Development of a potato tuber. Cell divisions and radial expansion of cells in cortex and pith in the subapical region of the stolon result in swelling, which eventually develops into a tuber. Modified from Simpson and Ogorzaly (1995).

tuber cells, however, they are arranged at random or longitudinally to the original direction of cell growth (for relationship of microtubular orientation to direction of growth, see Chapter 15).

Tubers normally form underground, but they can also be formed by aboveground parts, usually at axillary buds. Aerial tubers are generally small and contain chlorophyll. Indeed, any bud or shoot apex is capable of tuberizing. This ability of aerial shoots, or cut stem segments with an attached leaf, to produce tubers provides a convenient experimental system for studying tuberization under laboratory conditions.

3. ENVIRONMENTAL CONTROL OF TUBERIZATION

Various environmental factors regulate the onset and degree of tuber formation. They include photoperiod (day length), temperature, nutritive conditions, and, among hormones, gibberellins. All these factors interact in complex ways to induce or inhibit tuber formation.

Potato varieties differ in their photoperiodic requirement (critical photoperiod, CPP). *S. tuberosum* ssp. *andigena*, the subspecies of potato that is cultivated in the Andes, where the crop originated, requires short days (SDs) for tuberization. The same is true for the wild species of potato in Mexico, *S. demissum*. Both require a short CPP. Usually a succession of short days is required for induction, hence, the use of the term **inductive SDs**; also, more accurately, it is not the length of the day, but the duration of an uninterrupted night (dark period) that is critical. This is a phytochrome-mediated response involving phytochrome B (PhyB) and other members of the phytochrome family of photoreceptors (for photoperception and phytochromes, see Chapter 26). In contrast, exposure to noninductive **long days** (LDs) inhibits tuberization and, indeed, may promote the emergence of stolons aboveground and their growth as normal aerial shoots (Fig. 20-5). *Andigena* varieties cultivated in temperate regions of the world have been selected to tuberize under long days. Most potato varieties grown in temperate regions, however, belong to the subspecies, *S. tuberosum* ssp. *tuberosum*; they also are able to tuberize under long photoperiods. These varieties of *andigena* and *tuberosum* have long CPP—some will tuberize even under continuous illumination if other conditions are favorable. Nevertheless, shortening the photoperiod will intensify the induction of *tuberosum* varieties and increase the proportion of biomass partitioned to tubers. Under laboratory conditions, if

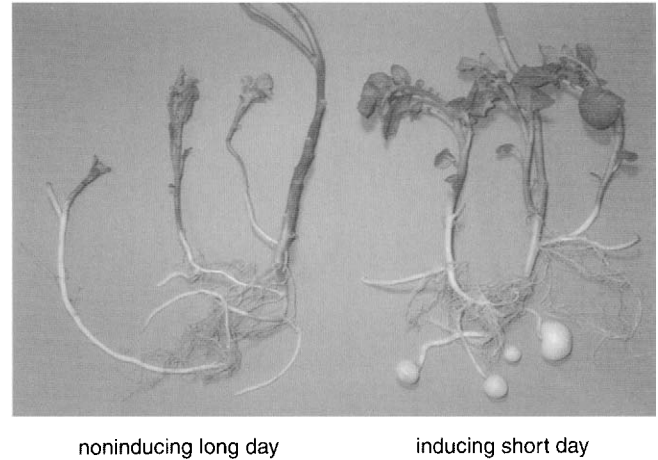


FIGURE 20-5 Potato (*Solanum demissum*) plants grown in long-day (LD, left) or short-day (SD, right) conditions. In SDs the stolons stay underground and the tips swell to form tubers. In LDs they grow aboveground and continue as shoots. From Jackson (1999).

the endogenous gibberellin content is depleted, tubers are formed irrespective of day length (see Section 5). They can also be formed under LDs in a typical cultivar of ssp. *andigena* if phytochrome levels are reduced in a transgenic plant by introducing the potato *PHYB* cDNA in an antisense orientation. How the reduced phytochrome level brings about this response is not clear. One possibility is that PhyB is involved in the production of an inhibitor under LDs, and depletion of its endogenous content reduces the concentration of the inhibitor (see Section 4). An alternative explanation may be that the depletion of endogenous phytochrome results in a reduced sensitivity of transgenic plants to photoperiod; under these circumstances, the transgenic plants set tubers irrespective of daylength, depending on nutritive conditions.

Temperature also affects tuberization. Cool temperatures (day temperature below 30°C and night temperature below 20°C) favor tuber induction, whereas high temperatures inhibit it.

When photoperiod and temperature are favorable for tuberization, factors that affect photosynthetic production, e.g., light intensity, age, or number of leaves, also influence the degree of tuberization. Under these conditions, mature plants with large photosynthetic area, given sufficient light, produce high tuber yields. The supply of sugar in high concentrations also results in tuberization in potato explants. In contrast, even under inductive SDs and permissive temperatures, a low light intensity or a decreased photosynthetic area reduces the extent of tuber formation.

A heavy application of nitrogen (e.g., N-rich fertilizers) promotes shoot growth and curtails tuber production. In hydroponic cultivation, under inductive

day length and temperature conditions, withdrawal of N may produce instant tuberization, whereas a supply of N may cause tubers to revert to stolons.

4. TUBER FORMATION IS REGULATED BY A GRAFT-TRANSMISSIBLE FACTOR

The photoperiodic signal is perceived in leaves, but tuberization occurs in underground stolons. Thus, it is reasonable to assume that some factor(s) is transmitted from the leaves to the stolons. To demonstrate the presence of such a factor, reciprocal grafts were made between scions and stocks taken from potato cultivars with vastly different photoperiod requirements for tuberization. It could be shown that tuberization occurred if the scions from an induced plant were grafted to a noninduced stock, but not in the reverse graft where noninduced leaves were grafted onto the stock of an induced potato plant (Fig. 20-6).

Interspecific grafts between members of the same family show that the factor can move between an induced potato scion and a noninduced potato stock, which are separated by a stem segment from eggplant or tomato (potato, eggplant, and tomato all belong to the same family, Solanaceae). Tobacco is another member

of Solanaceae. It has several cultivars that differ in their photoperiodic requirement for flowering. Some have an obligate requirement for inductive SDs, others for inductive LDs, and still others that flower under either SDs or LDs. Tobacco plants of different cultivars were exposed to inductive day lengths, and scions from these plants were grafted to noninduced stocks of potato to see their effect on tuberization. Data, presented in Table 20-2, show that the scion from an induced plant, kept under inductive conditions after being grafted, causes tuberization. In other words, the signal that causes tobacco plants to flower is the same signal that causes tuberization in potato.

Jerusalem artichoke (*Helianthus tuberosus*), a member of the family Asteraceae, also produces tubers under SDs. If leaves of sunflower (*Helianthus annuus*) plants, induced to flower under SDs, are grafted to stocks of a noninduced Jerusalem artichoke, they, similarly, induce tuberization in Jerusalem artichoke.

The transmissible substance moves through the phloem tissue both acropetally and basipetally. If phloem tissue is disrupted by heat girdling, transmission is stopped; in such a case, potato tubers may be formed from axillary buds above the girdle.

The identity of the transmissible substance is unknown. It could be a positive regulator that is produced under inductive conditions or, alternatively, it could be an inhibitor that is present under noninductive conditions, but whose concentration declines under inductive conditions.

5. HORMONAL REGULATION OF TUBERIZATION

Since the inducing factor is graft transmissible and mobile in phloem, attention has been focused for

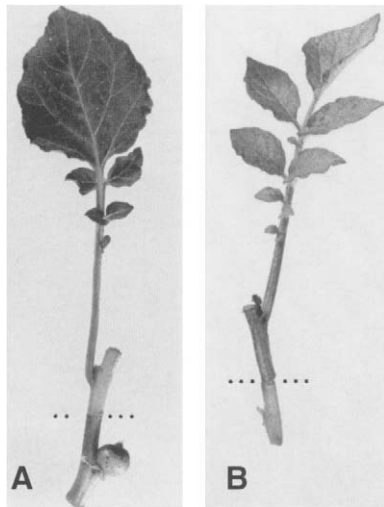


FIGURE 20-6 Grafts between an *andigena* clone, which is able to tuberize under long photoperiods, and a typical *andigena* with a short critical photoperiod. The lower leaf on each stock was excised at the time of cutting. Cuttings were taken from plants exposed to 20-h photoperiods and were maintained at the same photoperiod for 12 days after grafting. (A) Scion taken from an *andigena* clone that is able to tuberize under continuous illumination is grafted to a stock from a typical *andigena* stem. Note the sessile tuber. (B) A reciprocal graft. Graft union is indicated by dotted lines. From Ewing (1995).

TABLE 20-2 Flowering and Tuberization Responses in Grafts between Tobacco and Potato^a

Scion donor cultivar	SD		LD	
	Flowering response	Tuberization	Flowering response	Tuberization
Mammoth	+	+	—	—
Xanthi	+	+	+	+
Sylvestris	—	—	+	+

^aScions obtained from induced tobacco plants were grafted to noninduced potato stocks. Grafted plants were kept in LD or SD. Mammoth is a short-day species, Xanthi is a day-neutral species, and Sylvestris is a long-day species. +, positive response; —, negative response. Adapted from Ewing (1995).

many years on its being a hormone. Measurements of endogenous levels of hormones on transition from stolon to tuber, as well as exogenous applications of hormones, have been tried.

5.1. Gibberellins

Gibberellins, alone among the hormones, have been shown to have a definitive effect. They promote shoot and stolon growth and inhibit tuberization. This conclusion is supported by numerous experiments that indicate a reduced GA content or GA-like activity under conditions that promote tuberization (e.g., SDs) or, conversely, an increase in GA content under conditions that inhibit tuberization (e.g., exposure to LDs, high temperatures, low irradiance, or high N application). As expected, inhibitors of GA biosynthesis, such as CCC or ancymidol, promote tuber formation; in plants so treated, tubers may be induced even under LDs. Similarly, a dwarf mutant of *S. tuberosum* ssp. *andigena*, which has a partial block in GA biosynthesis, is able to tuberize in LDs. These data indicate that gibberellins and day length perception by phytochromes interact in the control of tuberization and that a reduction in GA content may relax the requirement for short days in tuber formation. This relationship between GA content and inductive day length for tuberization was elegantly confirmed by expressing the GA 20-oxidase gene in a sense or an antisense orientation in transgenic potato. An overexpression of the gene in a sense orientation caused the transgenic plants to require a greater

number of inductive short days to tuberize than the untransformed controls. Expression of the gene in an antisense orientation caused a decrease in GA content in the shoot apex and first leaves, shorter stems, and required a lesser number of inductive short days for tuberization. Tuber yield was also increased in the antisense line under SDs, but not LDs, which suggests that factors other than GA concentration are involved.

GA content and carbohydrate supply also interact; moreover, it is the GA content in the stolon where tuberization occurs that is important. Xu *et al.* (1998) used explants of potato and two concentrations of sugar: an 8% sucrose solution that induced tuberization and a 1% sucrose solution that was noninductive (Fig. 20-7A–C). The effect of inducing concentration of sucrose could be negated by increasing the concentration of GA ($GA_{4/7}$) in the medium from 0.01 to $1.0 \mu M$ (Fig. 20-7D–G). Measurement of endogenous GAs revealed the highest content at the stolon tip under noninducing conditions; these concentrations dropped sharply under inducing conditions. Switching between inductive and noninductive conditions could form a series of tubers in “chain.” These data are the clearest demonstration yet that gibberellins and sugar (or carbon) concentration interact at the tuber site in the decision to proceed with tuberization or not.

As mentioned earlier, potato tubers accumulate patatin, the most abundant protein in tubers. Gibberellins not only inhibit tuber formation, they also inhibit the expression of patatin genes.

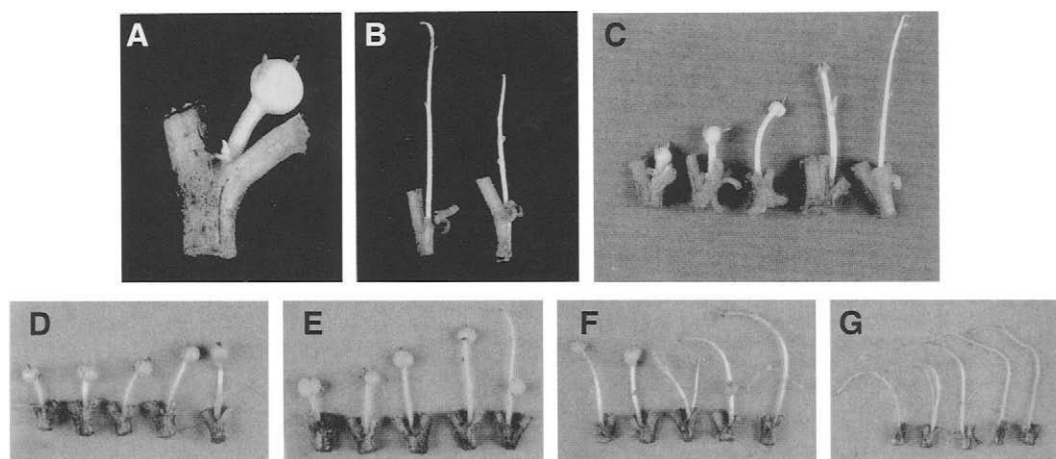


FIGURE 20-7 Development of single-node cuttings of potato plants grown *in vitro* on media after 10 days of culture. (Top) Tuber-inducing treatment with 8% sucrose in the medium (A); noninducing treatment with 1% sucrose in the medium (B); and a series of sucrose concentrations: 8, 6, 4, 2, and 1% from left to right (C). (Bottom) Effect of increasing concentration of GA on tuberization induced by 8% sucrose: 8% sucrose + $0.01 \mu M$ GA (D); $+0.03 \mu M$ GA (E); $+0.1 \mu M$ GA (F); and $+0.3 \mu M$ GA (G). From Xu *et al.* (1998).

5.2. Other Hormones

Evidence for other hormones being involved in tuberization is not strong. Several reports indicate high levels of cytokinins (CKs) in leaves of induced potato plants, but the connection with tuberization is not clear. ABA levels are reported to be higher in SDs, but an ABA synthesis mutant of potato, *droopy*, is able to form tubers. While a direct role of ABA in tuberization is doubtful, it may have a promotive effect because of its antagonistic action against GAs (see Chapter 24). Indoleacetic acid (IAA) and ethylene have also been implicated in tuberization, but the results are inconclusive. After harvest, potato tubers do not sprout for a finite period, which varies with the cultivar. This phenomenon, known as endodormancy, is thought to involve a complex interaction between endogenous ABA and other hormones, the details of which are obscure.

Tuberonic acid is the 12-hydroxy derivative of jasmonic acid (JA, see Chapter 12). Tuberonic acid and its glucosyl conjugate, as well as JA and MeJA, are reported to be potent inducers of tuberization in potato explants. However, the case for jasmonates and their derivatives playing a significant role in tuberization is weak.

S. demissum has an absolute requirement for short days for tuberization, but no differences in endogenous JA levels were observed in leaves of induced SD or noninduced LD plants. In the same plant, inhibition of JA biosynthesis by a derivative of salicylic acid (salicyl-hydroxamic acid) did not prevent tuberization under short days. Repeated sprays of JA on *S. tuberosum* ssp. *andigena* plants did not cause tuberization, even though it was taken up and transported in sufficient quantities to induce a systemic wound response. Jasmonates may, nonetheless, still be involved if they act as antagonists to GAs or if their content is high in stolons.

6. SECTION SUMMARY

Tuberization in potato is a multifactorial process in which day length, temperature, nitrogen, and sugar levels, as well as the content of bioactive gibberellins, play a role. Short days, cool temperatures, and high carbon/nitrogen (C/N) ratios promote tuberization, whereas a high GA content inhibits tuberization. However, these signals do not seem to act independently, rather they are integrated in the plant and the decision to tuberize is taken at the whole plant level. Thus, if the endogenous GA content is reduced by mutation or by treatment of wild-type plants with GA synthesis inhibitors or by antisense manipulation of GA biosyn-

thesis enzymes, the requirement for a short day length is relaxed. Day length perception occurs via members of the phytochrome family of photoreceptors, including phytochrome B. Transgenic plants in which the PhyB content has been reduced are able to tuberize under short or long days. The role of other hormones in tuber formation is sketchy and weak, although they may have an indirect role either by affecting the endogenous content of GAs or sensitivity to GAs or by affecting the nutrient (C/N) ratio in the plant.

SECTION III. SENESCENCE

1. SENESCENCE IN PLANTS AND PLANT ORGANS

Senescence, like growth and reproduction, is a normal phase of plant development, except that, in this case, death is the culminating event. Nearly all parts of a plant senesce, although which do and when are dictated by the genetic program of the plant. For instance, petals of many flowers (e.g., tulip, tomato) senesce and are abscised following pollination. Sepals, anthers, and styles may senesce after pollination or fertilization. In annuals, such as legumes and cereal crops, leaves senesce during seed or grain development. In perennials, leaves may show yearly cycles of senescence and abscission or, as in evergreens, senesce and abscise after 2–3 years, although not all at the same time. Some plants produce flowers once in their life and then die. These plants, known as monocarpic plants, include annuals (e.g., cereals, soybean, *Arabidopsis*); all biennials, (e.g., carrot, celery); and some perennials [e.g., century plants (most *Agave* spp.), bamboos].

Senescence-related changes also occur under adverse environmental conditions, e.g., drought, heat, nitrogen deficiency, light limitation, attack by pathogens, or onset of disease. Plants respond to these adverse circumstances by initiating changes that may result in leaf senescence and abscission, precocious seed development, and a reduced plant life span. A difference from natural programmed senescence is that these changes can be reversed if growth conditions become favorable. In contrast, natural senescence, which is part of the developmental program, occurs even under the most optimal environmental conditions.

2. HOW DO WE STUDY SENESCENCE?

Senescence has been studied in leaves (e.g., bean, *Arabidopsis*, maize) and in flower petals (e.g., carna-

tion, orchids). This chapter deals mainly with senescence in leaves. Although there are differences, the basic features are similar in petals. Physiological parameters used to measure senescence include a loss of chlorophyll and a decline in the photosynthetic rate. Both are relatively easy to measure. Yellowing is the visible manifestation of senescence, but by the time it is seen, the chlorophyll content is already down to about 50% of that in mature green leaf. Some researchers use detached leaves or dark treatment to induce senescence, but data in these cases do not always correlate with those obtained from intact plants under normal growth conditions.

3. SENESCENCE IS AN ORDERED SERIES OF EVENTS

Senescence is not a case of passive decay of structural and biochemical machinery of cells. Rather it is a highly regulated, ordered series of events in which organelles, membranes, and macromolecules are broken down and nutrients, i.e., amino acids, sugar, and minerals, are reclaimed for export out of the senescing leaf to be reused in other parts of the plant. Such reclamation is followed by abscission and/or death. Senescence in monocarpic plants, such as wheat or barley, is particularly dramatic because the entire resources of the plant are mobilized and redirected to support grain or seed development. In deciduous perennials, much of the nutrients reclaimed from leaves are stored as reserves in stem and root parenchyma for growth next spring in this chapter.

The first symptoms of senescence are a decline in the photosynthetic rate and an increase in the respiration rate. Other changes follow and include a breakdown of chloroplast membranes, loss of chlorophyll, and metabolism of proteins and lipids. Since it is important that certain cell organelles and tissues remain intact and functional until after mobilization is completed, organelles and tissues are degraded in a certain order (Fig. 20-8). Chloroplasts are rich reservoirs of proteins, RUBISCO and chlorophyll *a/b*-binding proteins, and membrane lipids. Some estimates are that these proteins and lipids account for >50% of protein and 70% of lipids in a mesophyll cell. Thus, chloroplasts are degraded first, while the mitochondria and peroxisomes remain functional. Nuclei also remain functional and transcriptionally active, and, although ribosomal degradation occurs, it is not completed until late in senescence. Guard cells in the epidermis and phloem tissue remain functional for gas exchange and transport, respectively, until the metabolic breakdown

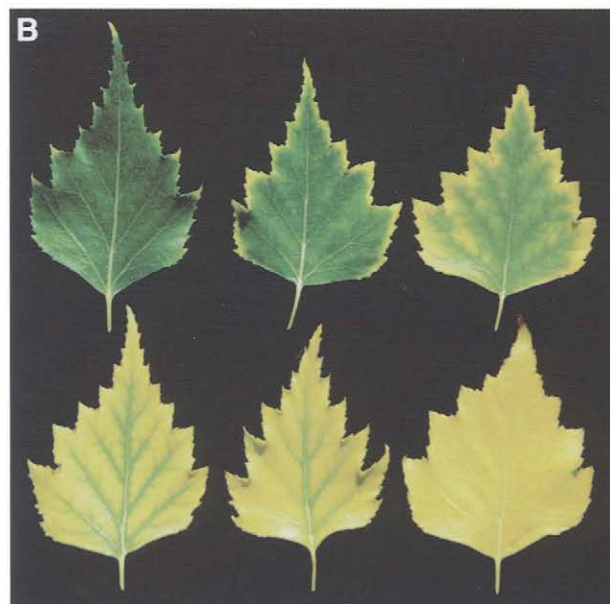
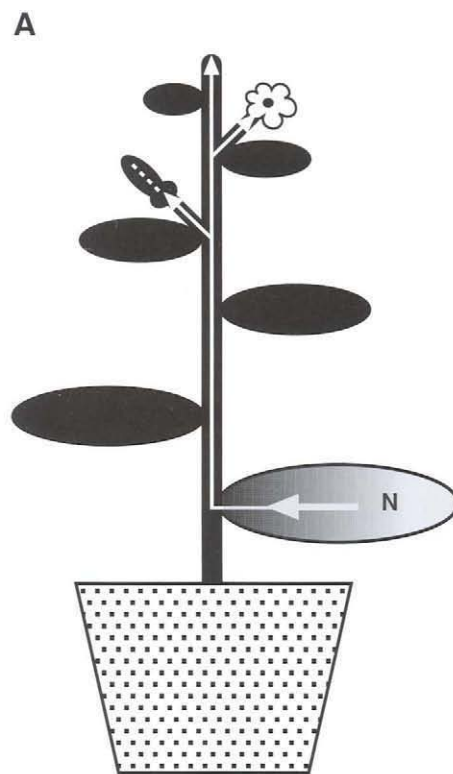


FIGURE 20-8 (A) Schematic illustration of nutrient recycling during leaf senescence. Some of the released nutrients (N) are transported to developing seeds and young organs at the shoot apex. From Gan and Amasino (1997). (B) Progression of leaf senescence in birch (*Betula* sp.). Senescence proceeds from leaf margins toward the center. Cells surrounding the vascular tissues senesce later to facilitate nutrient mobilization from adjacent senescing cells. Courtesy of Rick Amasino University of Wisconsin, Madison, WI.

of chloroplasts and export of metabolites is mostly completed.

Senescence is not altogether a degradative process. Many proteins, as well as secondary products, are synthesized during senescence. For instance, the loss of chlorophyll in autumn brings out the golden yellow color of carotenoids, but in many plants, leaves show red or orange coloration. Red or orange colors are due to the synthesis of anthocyanins, which are deposited in the vacuoles. Both for intercellular traffic and for movement of materials in and out of vacuoles, it is important that the plasma membrane and vacuolar membrane remain functional and selectively permeable until death.

4. DEGRADATION OF MEMBRANES

Turnover of membranes occurs regularly in healthy cells. It is accomplished by the removal of lipid metabolites, mostly free fatty acids, triacylglycerol, and a mixture of sterol and wax esters, and their assembly in the form of lipid-protein particles. These particles are shed by a process known as "blebbing" (Fig. 20-9). In senescing cells, blebbing is stopped and the particles accumulate between lipid bilayers, causing phase separation and ion leakage. Leaky membranes are degraded further. Lipid-protein particles occur in

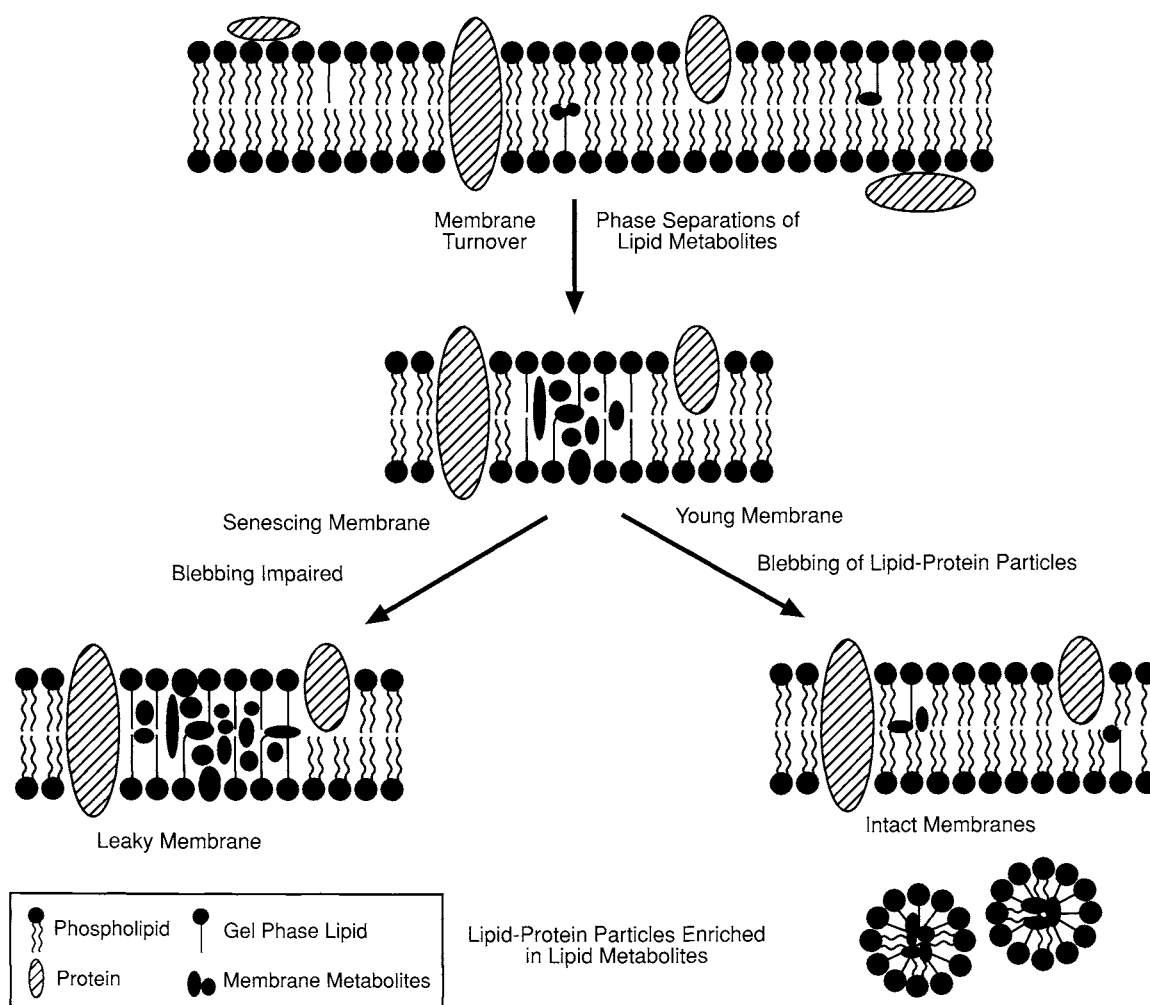


FIGURE 20-9 Diagrammatic illustration of blebbing of lipid-protein particles from membranes and the effects of its impairment with advancing senescence. In young membranes, metabolites formed within the membrane bilayer phase separate and are removed by blebbing of lipid-protein particles. In senescing membranes, blebbing is impaired, and the metabolites accumulate within the bilayer, causing the membrane to become leaky. From Thompson *et al.* (1997).

the cytosol and can be fractionated and examined chemically as well as under the electron microscope. Particles from chloroplast thylakoids are rich in galactolipids and pigment-associated proteins and, thus, can be distinguished from those from microsomal fractions.

5. GENE EXPRESSION DURING SENESCENCE

The breakdown of macromolecular components and transport of metabolites in senescing leaves requires the synthesis of many proteins (mostly enzymes). This can be shown by the use of inhibitors of transcription (e.g., actinomycin D) or translation (e.g., cycloheximide), which inhibit the synthesis of proteins and the progression of senescence. While many new genes are expressed, others, especially those involved in photosynthetic activity, are "turned off." Since the first demonstration of altered levels of mRNAs and their *in vitro*-translated products in senescing wheat leaves (Watanabe and Imaseki, 1982), a number of cDNAs and genes have been cloned from a variety of plants (e.g., *Arabidopsis*, asparagus, barley, *Brassica*, maize, tomato). These clones are often referred to as SAGs (for senescence-associated genes) and are defined as genes whose expression is upregulated during senescence. They have been classified in various ways by different authors in terms of their expression patterns during leaf development and their roles during senescence. For our purposes, they can be divided into two class: those expressed at a low basal level through most of

leaf development, but whose expression is upregulated with the onset of senescence, and those that are expressed only with the onset of senescence (Fig. 20-10).

The genes encode enzymes involved in the digestion of proteins and lipids, amino acid metabolism, as well as many defense-related proteins. Some of these are highlighted in the following.

5.1. Digestion of Proteins

Several genes encoding proteases (e.g., cysteine protease, aspartyl protease) have been isolated. Some of these proteases are similar to those expressed in germinated seeds for the digestion of reserve proteins (e.g., SAG2 from *Arabidopsis*; See1, for senescence-enhanced, from maize); some are similar to vacuolar processing enzymes (see Appendix 3), which may be involved in the activation of other enzymes (e.g., See2); and others bear a familial relationship to proteases involved in programmed cell death (PCD) in animal systems (e.g., SAG12 from *Arabidopsis*). SAG15 from *Arabidopsis* shares homologies with a bacterial protease. The specific functions/intracellular targets of these proteases are unknown. They do not seem to carry the target signal for chloroplasts and their role, if any, in hydrolysis of the chloroplast proteins is uncertain. If located in the cytoplasm, they could still hydrolyze those proteins after the breakdown of the chloroplast envelope.

In eukaryotic cells, including plant cells, proteins that have a short half-life or a rapid turnover are targeted for destruction by the ubiquitin/proteasome

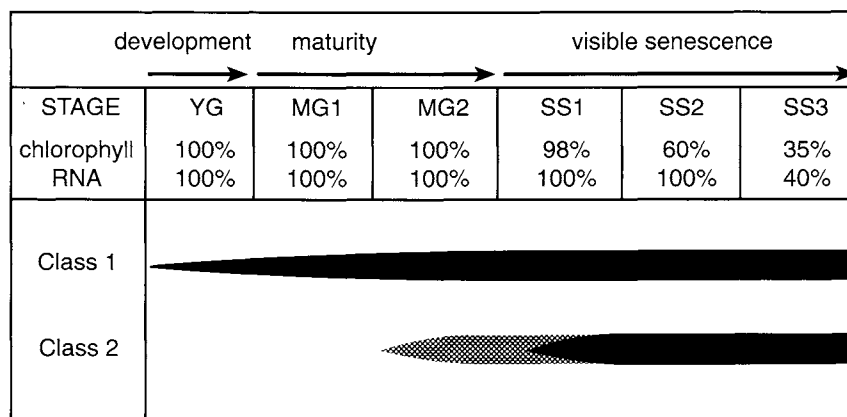


FIGURE 20-10 Differential expression of genes during leaf development. Class 1 genes are expressed at a low level in green leaves, but their expression is upregulated with the onset of senescence-related changes. Class 2 genes are expressed mostly from the onset of senescence until the death of the leaf. Stages of leaf development are shown as young green (YG), mature green (MG), and senescent (SS). Levels of chlorophyll and RNA are indicated as the percentage level in mature green leaves. Modified from Buchanan-Wollaston (1997).

pathway (see Chapter 22). Although a few clones encoding proteins associated with ubiquitination have been identified from senescing leaves of tobacco and potato, this pathway does not seem to play a major role in the degradation of proteins in senescence.

5.2. Digestion of RNA

Total RNA in senescing cells undergoes a decline by increased RNase activity, and several genes encoding RNases are upregulated in senescing leaves.

5.3. Genes Involved in Lipid Mobilization

Senescing leaves have a high respiration rate because a continuous supply of energy is needed to sustain the degradation of cell structures and the mobilization of nutrients. Fatty acids released by a breakdown of membranes yield acetate by β oxidation, which is utilized in respiration. Radiolabeling studies show that ^{14}C -labeled oleic acid supplied to green barley leaves is incorporated in galactolipids, the major lipid in chloroplast membranes, and is released as $^{14}\text{CO}_2$ during senescence.

Fatty acids are also utilized for the conversion to sucrose via a combination of glyoxylate cycle and gluconeogenesis (Fig. 20-11). In the glyoxylate cycle, which occurs in special microbodies called peroxisomes (or glyoxysomes), acetate is converted to four carbon acids. The four carbon products can then be converted to carbohydrates, particularly sucrose, via a reversal of glycolysis, a process known as gluconeogenesis. The sucrose can be used for respiration or exported and converted to other products. Genes encoding enzymes that catalyze steps in the glyoxylate cycle [e.g., isocitrate lyase (ICL), malate synthase (MS), and peroxisomal NAD-malate dehydrogenase] are upregulated in senescent leaves of cucumber and *Arabidopsis*. A gene encoding a phosphoenolpyruvate carboxykinase (PEPCK), which converts oxaloacetate to PEP, a step in gluconeogenesis, has also been reported.

Genes encoding a phospholipase D and a β -galactosidase, enzymes that may be involved in the hydrolysis of membrane phospholipids and galactolipids, respectively, are also upregulated.

5.4. Remobilization of Nitrogen

Nitrogen is translocated in the phloem stream mainly in the form of amides, glutamine and asparagine. Glutamine synthases (GS) are the principal enzymes that convert ammonia to glutamine. Two types of GS occur in plants: GS1 is located in cytosol and GS2 is located in plastids. During senescence, the

activity of GS2 decreases while that of GS1 increases. Several genes, presumably encoding GS1, are upregulated, which suggests that ammonia released from the catabolism of amino acids may be reconverted to glutamine in the cytoplasm for transport.

A summary of the metabolic pathways that may be involved in the remobilization of macromolecules during senescence, leading to an export of sucrose and nitrogen as glutamine and asparagine, is shown in Fig. 20-12.

5.5. Chlorophyll Breakdown

Chlorophyll breakdown proceeds by the loss of the phytol tail, which is cleaved by a chlorophyllase (chlase, EC 3.1.1.14) to yield chlorophyllide. Magnesium is removed from the porphyrin ring by an enzyme that dechelates it. The resulting pheophorbide is degraded further in a two-step process to give rise to a colorless, straight-chain tetrapyrrole, and the chlorophyll-binding proteins are released for degradation. The remaining chlorophyll catabolites are exported from the chloroplast, modified slightly in the cytosol, and then imported into the vacuole, where further degradation occurs.

5.6. Stress-Related Genes

Senescence of leaves renders them susceptible to attack by pathogens. It also generates oxygen-free radicals (e.g., superoxide [O_2^-], singlet oxygen [$^1\text{O}_2$]) and reduced oxygen species, such as hydrogen peroxide (H_2O_2), which can induce damage to cell structure and function unless scavenged by antioxidant mechanisms. Thus, many genes that encode pathogenesis-related (PR) proteins (e.g., a chitinase) are upregulated in senescing leaves. Other upregulated genes encode proteins for scavenging free radicals and detoxifying H_2O_2 . These proteins include superoxide dismutases, which convert the superoxide radical to H_2O_2 . H_2O_2 , in turn, is detoxified by catalases, peroxidases, and enzymes of the ascorbate-glutathione cycle. Genes encoding cytochrome P450-type monooxygenases and metallothioneins that scavenge metal ions are also expressed in senescing organs.

6. SENESCENCE-SPECIFIC MUTANTS

Senescence of a leaf is a coordinated degradation of a number of different functions; hence, no single gene is likely to regulate the entire syndrome of degradative

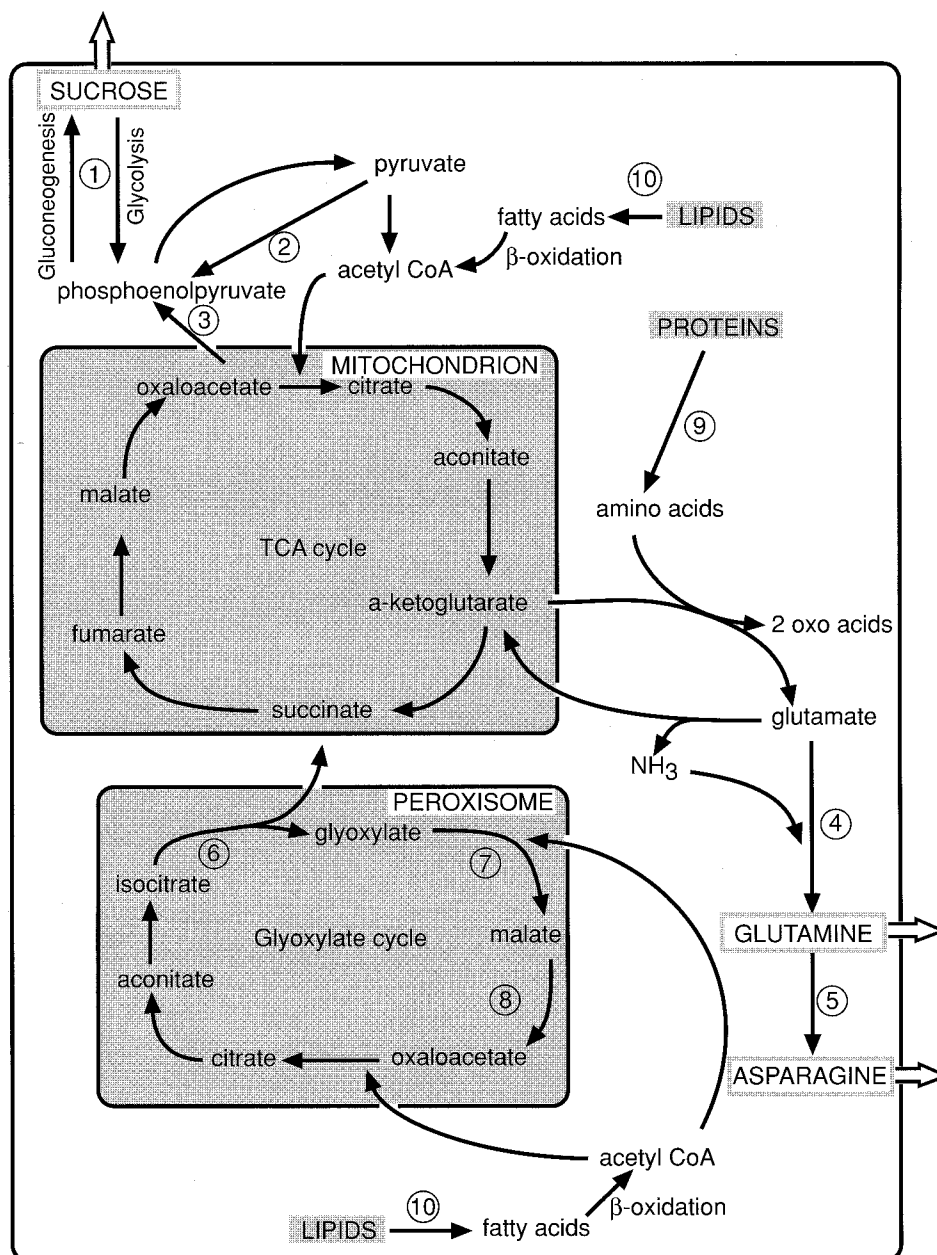


FIGURE 20-11 Interconnections between major metabolic pathways operative during senescence. Sugars are broken down in the glycolysis and tricarboxylic acid (TCA) cycle to provide energy. Acetyl-CoA derived from the β oxidation of fatty acids also enters mitochondria and participates in the TCA cycle. Acetyl-CoA may also enter the glyoxylate cycle. *Via* the glyoxylate cycle, acetate may enter mitochondria and condense with succinate to give rise to oxaloacetate and eventually sucrose by the process known as gluconeogenesis. Sucrose synthesized in this manner is used for respiration or is exported. Amino acids obtained from hydrolysis of proteins are transaminated and give rise to glutamine and asparagine, which are the major forms in which amino nitrogen is transported in the phloem tissue. Expression of genes encoding the following enzymes is elevated during senescence: (1) Fructose-1,6-bisphosphate aldolase and glyceraldehyde-3-phosphate dehydrogenase (required for both glycolysis and gluconeogenesis); (2) pyruvate orthophosphate dikinase; (3) PEP carboxykinase; (4) glutamine synthetase; (5) asparagine synthetase; (6) isocitrate lyase; (7) malate synthase; (8) NAD-malate dehydrogenase; (9) various proteases; (10) various lipid-degrading enzymes, such as phospholipase D and Δ^9 desaturase. Modified with permission from Buchanan-Wollaston (1997), © Oxford University Press.

changes. This partly explains the lack of single gene senescence-specific mutants, despite an intensive search for them in model plants (e.g., *Arabidopsis*, *Brassica*, maize). Several so-called “stay-green” mutations are known from plants, such as meadow fescue (*Festuca pratensis*), soybean (*Glycine max*), and bean (*Phaseolus vulgaris*). These mutants show an unusual separation between some components of the senescence syndrome. The pigment-protein complexes in the photosystems (light-harvesting units) and the thylakoid membranes containing them, as well as the associated carotenoids, are preserved, while the degradation of other parts of chloroplasts, including enzymes for carbon fixation such as RUBISCO, and cellular machinery proceeds normally. In soybean, some mutant lines show partial preservation of RUBISCO as well. The stay-green mutants tend to live longer than their wild-type counterparts, but eventually they all senesce. The mutant phenotypes appear to be regulated by alleles of nuclear genes, although in soybean, a cytoplasmic gene seems to be involved as well. Two mutant lines in *Arabidopsis* have also been isolated following ethylmethanesulfonate-induced mutagenesis. These mutants, described as *oresara1* and *oresara9* (*oresara* means “long-living” in Korean), showed delayed symptoms in several parameters, including chlorophyll content and the photochemical efficiency of PSII and the relative amounts of the large subunit of RUBISCO, as well as a delay in the onset of RNase and peroxidase activities, relative to the wild type. Efforts are continuing to clone the wild-type genes in *Festuca* and *Arabidopsis*. If several senescence-specific genes are cloned in a single plant, it may further our understanding of the regulatory processes that control senescence. It may also allow targeted mutagenesis, in which a single known gene is mutagenized, for a better understanding of its effect.

7. REGULATION OF SENESCENCE

Senescence is regulated by the developmental program of the plant, but it can be modulated by several hormones. Gibberellins, cytokinins, (CKs), and brassinosteroids (BRs) have been implicated in retarding senescence, whereas ethylene, abscisic acid, and jasmonates are reported to enhance senescence-related changes. Among these, CKs and ethylene are thought to play major, although opposite, roles. The effects of these two hormones are covered in more detail in the following, but it must be emphasized that, like other hormonal responses, their effects are seen only if the leaves are at the right developmental stage.

7.1. Hormonal Regulation

7.1.1. Cytokinins

Cytokinins delay leaf senescence but do not prevent it indefinitely and, once started, senescence progresses normally. Evidence comes from several sources: (i) Local application of a CK to mature green leaves delays senescence in the area where the CK was applied, while the rest of the leaf shows senescence (Fig 20-12A). (ii) Transformation of *Arabidopsis* or tobacco with the *ipt* gene from *Agrobacterium*, which encodes an isopentenyl transferase, can lead to an overproduction of cytokinins, and, among other effects, the transformed plants show delayed senescence (for CK biosynthesis, see Chapter 8 and Appendix 2). (iii) The *amp1* mutant of *Arabidopsis*, which overproduces cytokinins, also shows delayed senescence. Overproduction of cytokinin by *ipt* gene transformation or in *amp1* mutant has side effects that muddy the role of CKs in preventing or delaying senescence. (iv) To get a

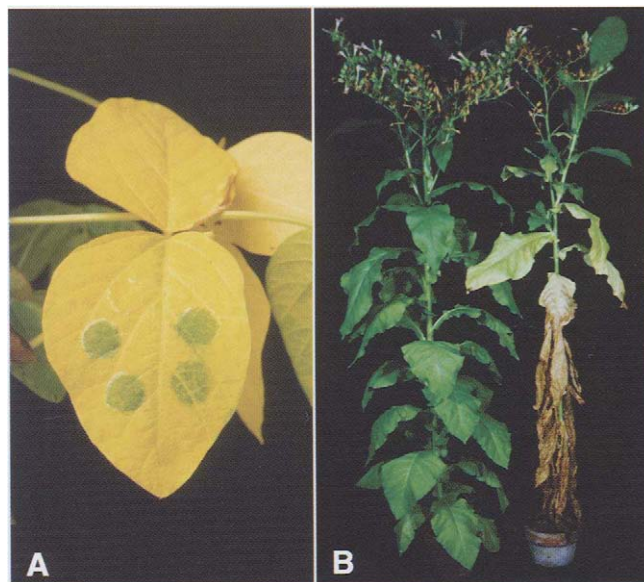


FIGURE 20-12 Retardation of senescence by cytokinins. (A) Local application of cytokinin to a soybean (*Glycine max*) leaf. A cytokinin solution was applied to the leaf, still attached to the plant, within lanolin rings. The two areas on the left side of the leaf, which remained green, received 9-(2-tetrahydropyranyl)-6-benzylaminopurine and the two areas on the right side received 9-(2-tetrahydrofurfuryl)-6-benzylaminopurine; both were applied once at 100 μ M. Both cytokinins used release free 6-benzylaminopurine (BA). Courtesy of David S. Letham. Australian National University, Canberra, Australia. (B) Inhibition of senescence in tobacco (*Nicotiana tabacum*) by the auto-regulated production of cytokinins. A *SAG12::ipt* construct was used to transform the plant on the left. An untransformed control is shown on the right. Plants are of the same age, but the transformed plant shows turgid green leaves, whereas the leaves have senesced in the nontransformed plant. Reprinted with permission from Gan and Amasino (1997), © Oxford University Press.

clearer picture, Richard Amasino and associates at the University of Wisconsin, Madison, used an ingenious method. They fused the promoter of the *SAG12* gene, which is induced specifically during the senescence of *Arabidopsis* with the coding sequence of the *ipt* gene. The construct *SAG12:ipt* was then used to stably transform tobacco plants using *Agrobacterium*. The transformed tobacco plants grew normally to the senescent stage and then showed significant retardation in leaf senescence compared to nontransformed plants (Fig. 20-12B). Photosynthetic capacity remained high, and more flowers were produced. The elegance of using a senescence-specific promoter is that as more CK is produced and senescence is halted, the *SAG12* promoter is turned off. This autoregulated production of CK obviated the side effects of cytokinin overproduction and showed clearly that CKs are involved in inhibiting senescence. The inhibition by CK is not permanent; it only delays senescence. Eventually, the transformed plants undergo senescence, which then proceeds normally. By using a *SAG12::GUS* construct for a double transformation, these researchers also demonstrated that CK production inhibited the expression of the *SAG12::GUS* construct, which suggested that CKs may be inhibiting the senescence program at the level of gene transcription. However, it is not essential to assume that CKs inhibit gene expression only at the transcriptional level or that all genes involved in senescence are regulated at the transcriptional level. As shown in Fig. 20-10, some genes are expressed more or less throughout leaf development, and it is possible that their greater expression during senescence is due to the greater stability of their mRNAs.

7.1.2. Ethylene

An exogenous application of ethylene to leaves causes epinasty. It also induces senescence-related changes in leaves and in flower petals. That ethylene has a direct role in these events is shown by the use of ethylene action inhibitors, such as silver thiosulfate, 2,5-norbornadiene, or 1-methylcyclopropene (1-MCP), which prevent senescence of cut flowers or detached leaves (see Fig. 11-12 in Chapter 11). Expression of genes encoding ACC synthase (ACS) or ACC oxidase (ACO) in an antisense orientation in transgenic plants results in delayed senescence, whereas the reverse is true if ethylene is overproduced by overexpression of an ACS gene. These effects of ethylene are brought about when the leaves or flowers are at the correct developmental stage. Young leaves or young flower buds do not show senescence-related changes, despite the presence of ethylene. In an *Arabidopsis* mutant

insensitive to ethylene, *etr1* (for ethylene-resistant), flowers senesced at about the same time as those in the wild type, but the rosette leaves, on average, had a 30% longer life span than in the wild type. However, once senescence started in the leaves, it progressed normally. These data suggest that the sensitivity of flower petals and foliage leaves to ethylene may be different or that there may be differences in the endogenous content of CKs between leaves and flowers. They also suggest that ethylene may not regulate senescence *per se*, but may modulate its timing.

Senescence of leaves and ripening of climacteric fruits share some common features: both involve chlorophyll degradation, an increased activity of hydrolytic enzymes, and ethylene production. However, the two are different processes, involving different sets of enzymes, and lead to different end results. That ethylene action is specific to a developmental state is shown by fruits as well. Whereas mature green tomatoes respond to ethylene, young immature tomato fruits show no response.

7.1.3. Other Hormones

While an overwhelming body of literature indicates that cytokinins retard senescence, there are also many, although less substantiated, reports that gibberellins and brassinosteroids retard leaf senescence. Evidence consists mainly of a reduced rate of chlorophyll loss in senescing leaves when sprayed or supplied with bioactive GAs or BRs. Also, the BR-deficient mutants show an earlier onset of senescence.

Similarly, while ethylene is known to enhance senescence in leaves and flowers of many plants, ABA and jasmonates are also reported to accelerate senescence. In certain plant families (e.g., Brassicaceae, Convolvulaceae, Orchidaceae, Caryophyllaceae), ethylene accelerates the senescence of flowers, whereas in certain other families (e.g., Amaryllidaceae, Liliaceae, Iridaceae, Asteraceae), it has no effect—floral death is neither hastened by physiological levels of ethylene nor is it prevented by the use of its synthesis or action inhibitors. In daylily (*Hemerocallis* sp.), a member of Amaryllidaceae, flowers senesce in a rapid, highly predictable fashion without any external stress, and cell deterioration is obvious just 24 h after bud opening. In this plant, exogenous ABA hastens senescence-related ion leakage and peroxidation of lipids in isolated petal segments; it also enhances RNase and proteinase activities (Fig. 20-13). Moreover, the amount of endogenous ABA shows an increase during senescence.

Jasmonates are known to promote the senescence of oat leaves and chlorophyll degradation in barley

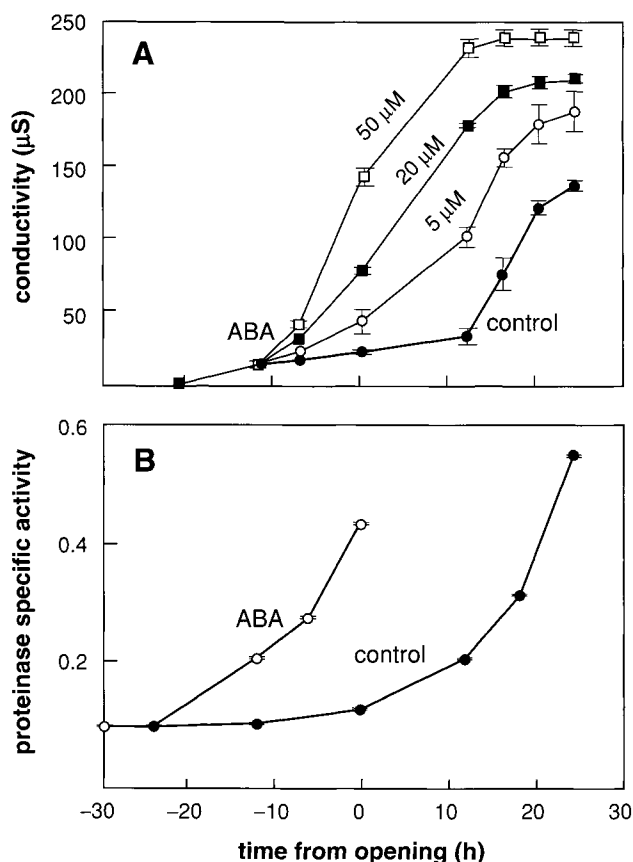


FIGURE 20-13 Effect of exogenous ABA on ion leakage and proteinase activity in daylily (*Hemerocallis* sp.) petals. (A) Ion leakage from eight petal discs, 8 mm in diameter, floating on water (control) or on 5, 20, or 50 μ M ABA was determined by solution conductivity. (B) Proteinase activity in petals incubated in water (control) or in 100 μ M ABA. Data show that ion leakage and proteinase activity are both enhanced or accelerated in petals exposed to ABA. Values represent the means and SE from three independent experiments. From Panavas *et al.* (1998).

leaves. Genes encoding chlorophyllase, the enzyme that catalyzes the first step in chlorophyll degradation, have been cloned from *Chenopodium album* and *Arabidopsis thaliana*; and one gene in *Arabidopsis* (*AtCHL1*) is rapidly induced by MeJA. In barley leaves, jasmonate levels increase during senescence-induced lipid degradation and induce the expression of many senescence-related genes, including a cysteine protease, a proteinase inhibitor, and a 4-hydroxyphenylpyruvate dioxygenase, which catalyzes a step in the synthesis of tocopherol, a potent antioxidant. Ethylene also induces these genes, and the two hormones together show additive effects, but those from jasmonates can be curtailed by the treatment of tissue with an inhibitor of JA biosynthesis, acetylsalicylic acid.

In summary, several hormones, CKs, GAs, and BRs, retard the onset of senescence, whereas ethylene, ABA,

and JAs accelerate the senescence-related changes. How these hormones modulate the senescence program is unknown, but they do so within the context of the developmental program.

7.2. Developmental Regulation

A decline in the photosynthetic rate is sometimes regarded as the signal for leaf senescence to commence. Cytokinins are transported from roots into shoots, and a decline in cytokinin levels in xylem sap has been correlated with an onset of leaf senescence. However, such a decline in the photosynthetic rate or in CK content is probably already the result of some other signal. The *knotted1* (*kn1*) gene in maize and its homologues in maize, *Arabidopsis*, and some other plants encode homeodomain-containing transcription factors, which are expressed in the shoot apical meristem and maintain it in a permanently meristematic state (see Chapter 4). Overexpression of *kn1* results in a variety of phenotypes that are similar to those caused by the overproduction of cytokinin. These include alterations in leaf shape, loss of apical dominance, and the production of ectopic meristems on leaves. In addition, leaf discs from *kn1*-overexpressing tobacco plants regenerate shoots in the absence of cytokinin in culture medium. In an effort to understand the developmental regulation of senescence, *kn1* was expressed in transgenic tobacco under the control of the senescence-specific *SAG12* promoter from *Arabidopsis*. The transformed plants developed normally and, as expected with the *SAG12* promoter, *kn1* mRNA was expressed only in old, but still green, leaves that were chronologically at an age when their counterparts in nontransformed plants were beginning to senesce. In these leaves, senescence, as measured by a loss of chlorophyll, was markedly delayed relative to nontransformed controls. Also, these leaves had a considerably higher (~15-fold higher) cytokinin content than those in nontransformed plants (Fig. 20-14). Most of the increase in cytokinin was due to zeatin, dihydrozeatin, and their ribosides. Thus, it seems that regulatory genes, such as *kn1*, may exercise a negative control over senescence by maintaining high cytokinin levels in the leaf, and a relaxation in their activity may be a signal for senescence-related changes to commence.

8. SENESCENCE AND PROGRAMMED CELL DEATH (OR APOPTOSIS)

Programmed cell death (PCD) is a process of selective elimination of cells and tissues that are no longer

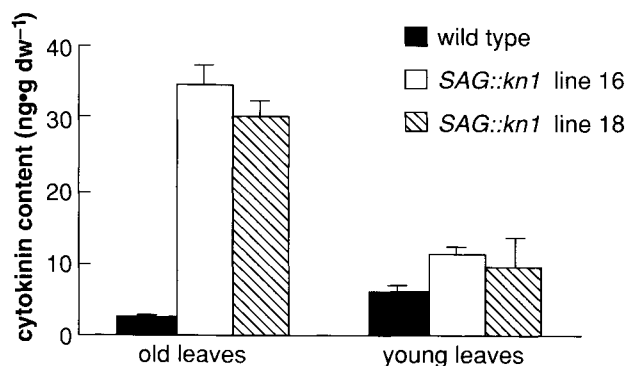


FIGURE 20-14 Cytokinin content in leaves of wild-type and *SAG::kn1*-transformed lines of tobacco. Data for two independent transgenic lines (16 and 18), as well as for “old” and “young” leaves, are presented. “Old” leaves showed an accumulation of *kn1* mRNA. They also showed a much greater difference in cytokinin content between the transformed lines and much greater wild type than the young leaves. Reprinted with permission from Ori *et al.* (1999), © Oxford University Press.

wanted. PCD occurs in both animals and plants. Examples in plants include degeneration of the suspensor during embryo development in angiosperms; degeneration of potential megaspores in favor of a few or one functional megaspore in *Selaginella*, gymnosperms, and angiosperms; elimination of stamen primordia or, alternatively, gynoecia in flowers of maize, which results in the production of unisexual flowers; differentiation of tracheids and vessel elements in xylem; and the hypersensitive reaction in plant defense, which eliminates whole blocks of cells at the site of infection to prevent a further spread of pathogen.

Senescence of leaves and flowers has also been considered PCD, but is controversial. PCD in animal cells, where it has been studied more, involves cytoplasmic and nuclear shrinkage, and fragmentation of nuclear DNA into characteristic “laddered” fragments, which are multiples of 180 bp. Many proteases are synthesized. Proteases of the interleukin-1 β -converting enzyme (ICE) family bring about cell condensation and shrinkage, and endonucleases cause fragmentation of nuclear DNA. The basic leucine zipper transcription factor, *ces-2* (for cell death specification), is necessary for PCD in some cell types. Reactive oxygen species are produced and can trigger PCD. Senescence of leaves involves some proteases, such as SAG12, which bear a familial relationship to the ICE protease; and reactive oxygen species are produced. But fragmentation of nucleus and cytoplasm and the laddered fragmentation of nuclear DNA have not been recorded in plant leaves undergoing senescence.

9. SECTION SUMMARY

Senescence of plant organs or whole plants is a natural phase of development. While it can be brought on by adverse environmental conditions, disease, or pathogen attack, it occurs at the genetically determined time in otherwise healthy plants despite under the most optimal growth conditions. Senescence-related changes progress in an orderly manner. Organelles, such as chloroplasts, with their vast reservoirs of cell proteins and lipids, are degraded first, releasing amino acids, fatty acids, and minerals for further metabolism and/or transport out of the senescing organs. These nutrients are transported to other parts of the plant, especially developing seeds and, in deciduous perennials, to storage parenchyma cells in stems and roots. Many genes encoding proteases, RNases, enzymes involved in lipid metabolism, generation of sucrose from fatty acids, and generation of phloem-transport-specific amino acids are expressed in senescing leaves. In addition, because senescent leaves are susceptible to pathogen attack and senescence causes the production of reactive oxygen species, various pathogenesis-related proteins, as well as antioxidants, are synthesized. The developmental factors that cause senescence-related changes to begin are not known. Also, mutants specific to discrete steps in senescence are rare. Considerable data support the postulate that a high cytokinin content in the leaf delays senescence, whereas production of or exposure to ethylene accelerates senescence. However, data from transgenic plants with altered levels of cytokinins or ethylene indicate that these hormones retard or hasten senescence, respectively, but do not alter the normal progression of senescence once it starts. These two hormones may not be the only hormones regulating senescence. There is evidence that ABA may hasten the senescence of flowers in some plants. Senescing organs are abscised and/or die; senescing whole plants also die, but senescence seems to differ from programmed cell death.

SECTION IV. ABSCISSION

1. ABSCISSION: THE PHENOMENON

Shedding of leaves in deciduous trees in autumn is a dramatic phenomenon, an aesthetic delight (Fig. 20-15), but for the plant it is a process by which it gets rid of organs that are no longer needed. Abscission usually follows senescence of the organ, but senescence is not essential for an organ to be abscised. The shed



FIGURE 20-15 Leaf fall in tress of *Ginkgo biloba*.

organs include leaves, flowers, floral parts, mature fruits, and whole branches that are old or decaying. Shedding of otherwise healthy organs occurs as part of a natural thinning process, whereby excess vegetative or floral buds, young fruits, or unpollinated ovaries or flowers are shed.

Abscission is a controlled process that is initiated in advance of the actual shedding of the organ. In most cases, an abscission zone (AZ) is formed in a region between the organ to be shed and the body of the plant. The AZ has two functions: to facilitate separation by the hydrolysis of wall materials between defined cell layers and to initiate the synthesis of materials that protect the body of the plant from water loss and from infection by microorganisms.

2. THE ABSCISSION ZONE

The location of the AZ varies widely in different organs or the same organ of different plants. In

leaves, the AZ is usually formed at the base of the petiole near its junction with the stem. It is often characterized as a band of small, densely cytoplasmic cells arranged in rows from about 5 to 50 layers in thickness (Fig. 20-16). The AZ may be distinguished early in relatively young leaves that are still expanding. Cells destined to form the AZ usually fail to expand and vacuolate along with the neighboring cells. Differentiation of lignified fibers or sclereids may be relatively sparse in the zone, but, otherwise, cells in the AZ seem to be organized along the same tissue pattern, i.e., epidermis, cortex, vascular tissues, and pith, as in the rest of the petiole. Cell separation does not occur throughout the entire AZ, but is typically confined to a one to five cell wide **separation layer** at its distal end, i.e., the end farther away from the stem. Cells in the separation layer synthesize and secrete wall hydrolases between two layers of cells, thus dissolving the middle lamella and disrupting the primary wall. The fracture occurs between the two cell layers all across the width of the petiole, and all cells participate except the dead tracheary cells, vessel elements, and tracheids, which are broken mechanically. Before the fracture occurs, a few layers

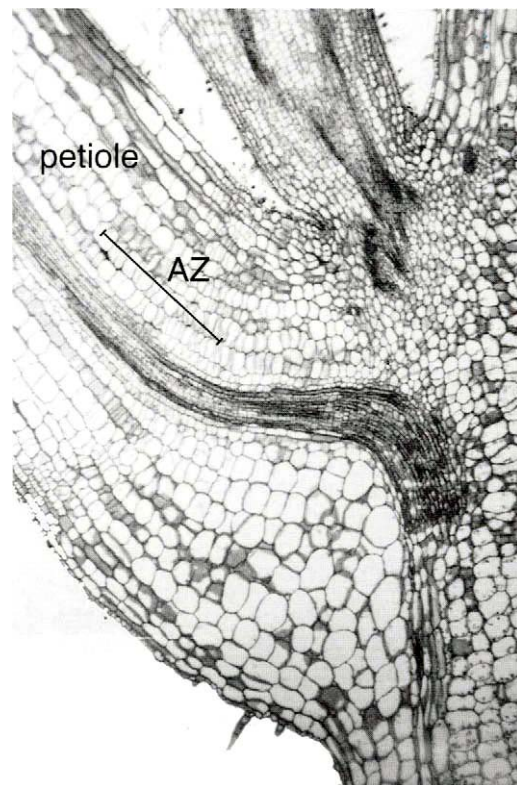


FIGURE 20-16 Abscission zone (AZ) in a petiole of a *Coleus* leaf. The AZ in this plant occurs near the base of the petiole.

of cells on the proximal side of the separation zone, i.e., toward the stem, form the **protective layer**. The cells in the protective layer may expand, balloon out, and show other morphological changes. Their main function is to synthesize defense-related proteins, which protect the freshly exposed surface from pathogenic infection. Eventually, these cells may form a periderm, which protects the stem tissues from water loss. Loss of water through the broken ends of xylem vessels and tracheids is prevented by the formation of tyloses. Tyloses are outgrowths from xylem parenchyma cells that grow into the lumen of tracheary cells through pits, balloon out, and occlude the cell interior. In other instances, resins, gums, and other secretions may plug up the interior of the tracheary cells.

An AZ is formed and functions similarly in floral peduncles, although the location of the zone varies. It may be formed near the base of the pedicel, in the middle, or near the top below the gynoecium.

In most cases studied, the AZ arises in cells that were laid down during organ development. These are sometimes referred to as "primary" AZ. However, the formation of AZ by new cell divisions, giving rise to "adventitious" AZ, is not excluded. Abscission of branches is poorly studied, but an AZ is probably formed adventitiously by localized cell divisions in parenchyma cells of the cortex, secondary xylem and phloem, and pith. The further differentiation of separation and protective layers

probably occurs similarly to that in petioles and pedicels.

3. HOW DO WE STUDY ABSCISSION?

Abscission of leaves can be studied *in planta* by monitoring the rate of abscission, i.e., the number of leaves shed per unit time, when exposed to an environmental or hormonal stimulus. However, these studies are complicated because, in nature, abscission usually follows senescence, and many of the same factors that regulate senescence also regulate abscission. To separate the two processes, the abscission of leaves is generally studied under conditions when senescence is not a factor. It has been known for some time that if the blade of a mature leaf is removed, a process named "deblading," the debladed petiole, depending on the species, lasts a few days on the plant, but then abscises. This property lends itself to a study of abscission *in vitro*. Stem segments, each with one or more debladed petioles, can be used under controlled conditions to monitor the effect of hormones (or their inhibitors) on abscission (Fig. 20-17). To lend greater objectivity, a machine that records the break strength of a petiole, or pedicel, is used. The principle is that the greater the abscission (or the separation process), the lesser the force (g/unit area) required to affect the separation. Similar experiments can be done with the pedicels of flower or fruits.

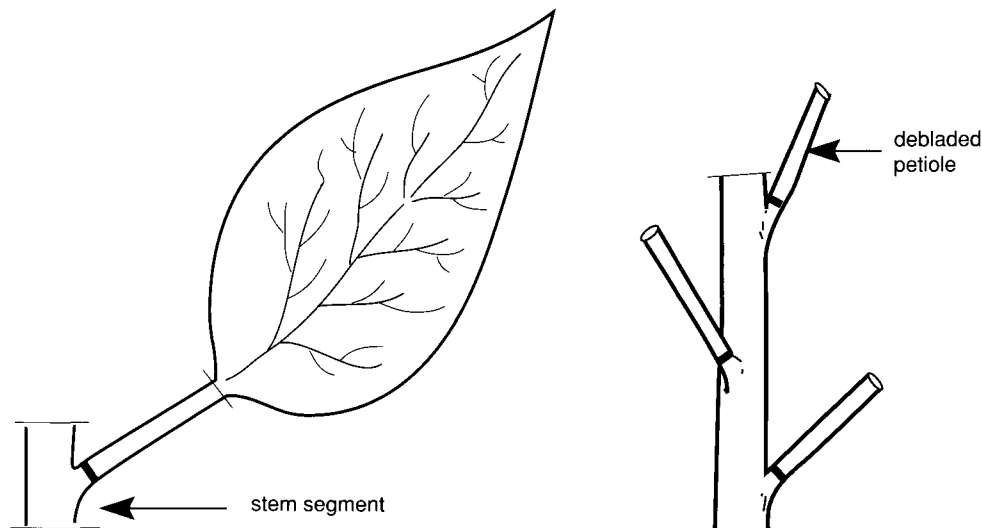


FIGURE 20-17 Bioassay for abscission in tomato. Explants with a single debladed petiole, or longer stem segments with three to four debladed petioles, are used. The assay lends itself to use of a large number of nearly identical samples. AZ is represented by the thick line at the base of petioles.

4. KINETICS OF ABSCISSION

In vitro experiments using explants allow a study of the kinetics of abscission and permit a comparison of such kinetics in different organs or plants. For any single organ, e.g., bean leaf, provided the leaf is of an appropriate age (see Section 5.1), there is a lag period during which the break strength does not change appreciably; the lag phase is followed by a period of rapid decline in break strength (Fig. 20-18). Data on abscission kinetics from a variety of materials show that the time interval between induction of abscission by a stimulus and completion of separation varies widely among different materials and plants. For most leaves and fruits, abscission is completed between 10 and 60 h, whereas floral structures are generally shed much more rapidly, within 2–8 h.

Explants also permit a more precise measurement of the effects of various hormones on the abscission process and genes expressed during abscission.

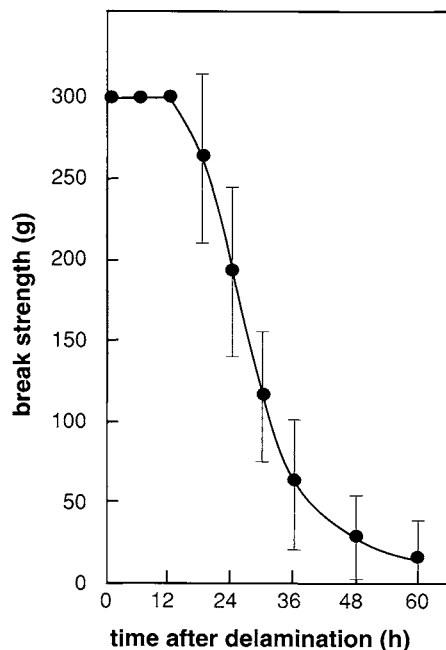


FIGURE 20-18 A plot of ethylene-induced abscission of a petiole in bean (*Phaseolus vulgaris*). Break strength is the force in grams required to affect a separation. Before 18 h the values are notional because the fracture is irregular and does not truly represent the strength of the separation layer. A lag period of 12–18 h is followed by a rapid decline in the force required to affect the separation. $n = 70$ zones. Modified with permission from Durbin *et al.* (1981), © Blackwell Science Ltd.

5. HORMONAL REGULATION OF ABSCISSION

5.1. Ethylene Is a Natural Regulator of Abscission

Ethylene has been known for a long time to promote leaf, flower, and fruit abscission. Whole plants exposed to ethylene gas show enhanced rates of leaf or flower abscission. Measurements of ethylene production rates in abscising organs, such as flower petals and leaves, indicate that they are sufficiently high to cause abscission. If such production is curtailed by the use of ethylene synthesis inhibitors (e.g., aminoethoxyvinylglycine, Co^{2+}), the abscission rate is reduced. In experiments using explants, ethylene accelerates the rate of abscission, and inhibitors of ethylene action (e.g., silver thiosulfate, 2,5-norbornadiene), used along with ethylene, reduce the rate significantly. Ethylene-insensitive mutants in *Arabidopsis* show delayed senescence and delayed abscission of flowers and leaves. Thus, there is abundant evidence that ethylene is a regulator of abscission.

For ethylene to cause abscission, however, leaves or flowers must be at the right developmental stage. Leaves are not sensitive to abscission by ethylene at all times and their sensitivity changes with age. Young growing leaves have some of the highest ethylene concentrations, but young leaves do not abscise; whereas mature leaves abscise at much lower ethylene concentrations.

It should also be noted that ethylene is not the only regulator of abscission. According to some estimates, 60% of plant species investigated show no effect of ethylene on abscission or a poor correlation between ethylene production and abscission, which leaves the possibility open that other hormones mediate their abscission.

5.2. Auxin Inhibits Ethylene-Induced Abscission

The fact that debladed petioles abscise suggests that some factor produced in the leaves moves from the blade to the petiole, preventing abscission. Experimental evidence suggests that this factor is IAA. Auxin application to the cut end of the debladed stump, or distally to the AZ, inhibits abscission (Fig. 20-19, site 1). In order to be effective, however, auxin must be applied at the beginning of the lag phase. If application is delayed until later in the lag phase, auxin may have little or no effect on arresting abscission. These data

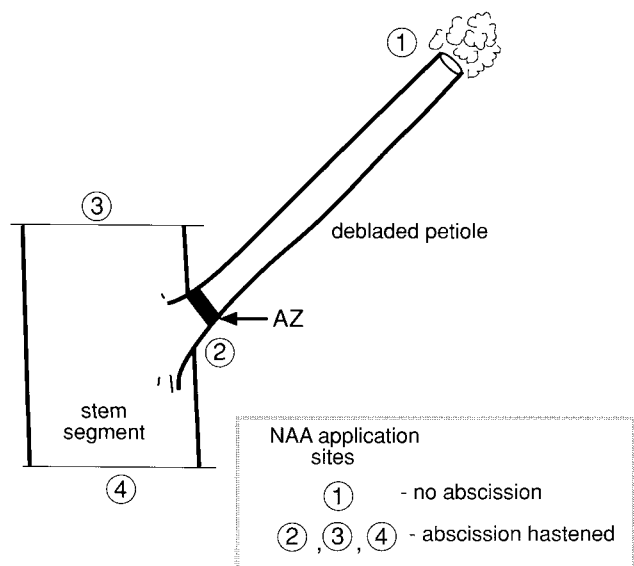


FIGURE 20-19 Effect of applied auxin on the abscission of debladed petioles. IAA applied at the distal end of the petiole (site 1) retards abscission; but applied at either the proximal end of the petiole (site 2) or the upper or lower end of the stem segment (sites 3 or 4) hastens abscission.

suggest that auxin somehow prevents the AZ from becoming sensitive to ethylene.

Interestingly, if auxin is applied to the proximal side of the AZ, near the petiole junction with the stem (or the cut basal or apical end of the stem segment), it enhances the rate of abscission (Fig. 20-19, sites 2, 3, or 4). A good explanation for this phenomenon is not at hand, but it has been suggested that in the intact leaf there is a gradient of auxin from the leaf tip, across the blade and AZ to the base of the petiole; and that it is the gradient rather than the absolute amount of auxin that is critical for maintaining the AZ in an ethylene-insensitive state. A proximal application of auxin reverses this gradient and, thus, stimulates abscission. Alternatively, a proximal application of auxin may stimulate ethylene synthesis without altering the effective gradient of auxin in the AZ (for auxin-induced ethylene biosynthesis, see Chapter 11).

6. NEW PROTEIN SYNTHESIS IS REQUIRED FOR ABSCISSION TO OCCUR

Many new proteins are synthesized during the abscission process. This can be demonstrated by the use of inhibitors of transcription or protein synthesis, such as actinomycin D or cycloheximide. If these inhibitors are supplied early in the lag phase, they retard ethyl-

ene-induced abscission or prevent it altogether. If they are given at later times, they have little effect. It has also been known for a long time that cell wall hydrolases, such as endo-1,4- β -glucanases (cellulases) and polygalacturonases (PGs), are synthesized, and their levels increase dramatically in the AZ on induction of abscission by ethylene. For example, the ethylene-induced petiole abscission in bean is correlated with a 40-fold increase in cellulase activity. Similar increases in cellulase and/or PG activities have been recorded in tomato. These increases in protein or mRNA levels (see Section IV,7 below) below are correlated with the drop in break strength. Because the synthesis of these wall hydrolases is inhibited by auxin, a reasonable hypothesis is that, in a debladed petiole, a lag period is required for auxin concentrations to drop below inhibitory levels such that the synthesis of hydrolases can begin. Some other proteins whose synthesis is not inhibited by auxin, such as pathogenesis-related (PR) proteins, may be synthesized much earlier.

7. GENE ACTIVITY DURING ABSCISSION

Two main classes of genes are expressed in the abscission zone. One class encodes cell wall hydrolases, endo-1,4- β -glucanases (EGases or cellulases), and PGs. It will be recalled that these enzymes are also synthesized during cell and organ growth and during ripening of fruits (see Chapters 15 and 17; also Box 15-2 in Chapter 15). Cellulases are encoded by multigene families. The expression of different isoforms of these enzymes is an active area of research, but it appears that different isoforms of cellulases participate in cell growth in vegetative tissues and fruit enlargement on the one hand and fruit ripening and abscission of organs on the other. Cellulase genes expressed during the abscission of leaves, flowers, and fruits in several plants (e.g., bean, tomato, avocado) encode cellulases that have a *pI* of 9.5, whereas cellulase genes expressed during vegetative growth or fruit enlargement have a *pI* of 4.5. The 9.5 *pI* cellulases are induced by ethylene and repressed by auxin, whereas the 4.5 *pI* cellulases are induced by auxins. Moreover, in bean petioles, where the 9.5 *pI* cellulase has been most studied, the enzyme is expressed in the AZ, specifically in two to three cell layers and vascular tissues of the separation zone (Fig. 20-20). It is synthesized *de novo* because radioimmunoassays provide no evidence of a preexisting inactive enzyme prior to ethylene treatment.

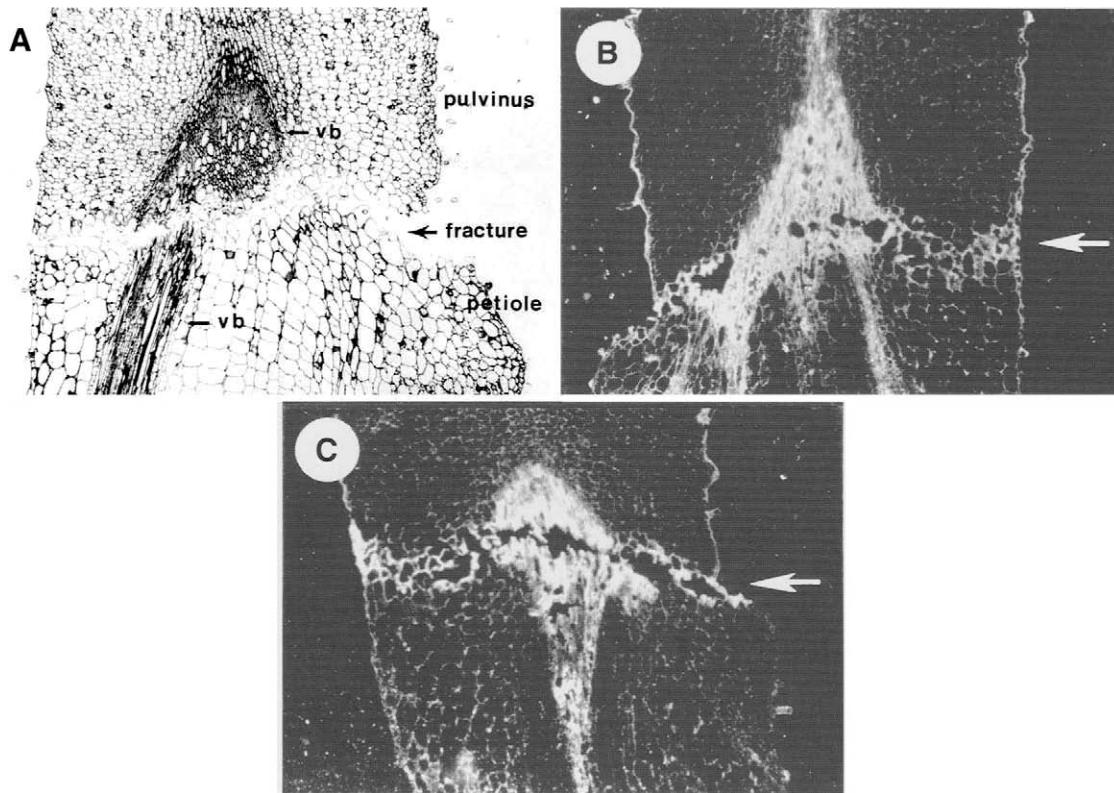


FIGURE 20-20 Longitudinal sections from the ethylene-induced bean leaf abscission zone (AZ). (A) Bright-field micrograph of a toluidine blue-stained section. vb, vascular bundle. (B and C) Detection of cellulase mRNA in the AZ by *in situ* hybridization. Two sections hybridized to ^{35}S -labeled antisense RNA transcript of a bean abscission-specific cellulase cDNA followed by autoradiography. Arrows point to line of fracture. Dark field photographs. The hybridization signal in these autoradiographs is apparent by bright, light-reflecting silver grains. From Tucker *et al* (1991).

PG cleaves the 1,4 link in polygalacturonic acid (PGA) in the pectic matrix. Several PG genes have also been isolated from tomato. They are specifically expressed during the abscission of leaves and flowers and, like AZ-specific cellulases, show sequence similarity to PG genes expressed in ripening fruits. A different class of PG genes are known and encode isoforms that may be more involved in pollen growth.

Pectin methyl esterase (PME) was implicated in the past in leaf abscission, but subsequent research has shown no consistent relationship between abscission and PME activity.

The other class of enzymes expressed in AZ includes defense- or pathogenesis-related proteins such as β -1,3-glucanases, peroxidases, and chitinases. These proteins have been proposed to play a role in protecting the fracture surface from pathogenic attack once cell separation has taken place. Other defense-related proteins include antioxidants such as metallothionein and Win4 protein belonging to the wound-induced (Win) gene family. Genes for many of these proteins have been cloned. Their mRNAs are expressed in the

AZ in cells of the separation layer before actual separation occurs, and they are induced by ethylene.

8. FORMATION OF PERIDERM

The formation of periderm in the AZ tissue proximal to the stem is a process distinct from abscission, as it involves synthesis and deposition of new wall substances, suberin and cutin. Its formation is probably initiated at about the same time as the production of wall hydrolases and defense-related proteins. However, the phenomenon has not been well studied and there is little recent information on this topic.

9. CORRELATION OF SENESCENCE AND ABSCISSION

In nature, senescence of an organ and its abscission usually go together. As shown elsewhere in this chap-

ter (Section III), cytokinins retard senescence and ethylene promotes it, but there is no clear evidence that auxin has a role in senescence prevention. However, in abscising organs, while ethylene still promotes abscission, it is auxin that retards it, and there is no clear evidence that CKs have a role in the prevention of abscission. An explanation for this apparent paradox probably lies in the system used to study abscission. As mentioned earlier, to separate the twin phenomena of senescence and abscission, explants are taken from organs that are not yet senescent, but nonetheless are responsive to ethylene. A composite picture that emerges, therefore, is that young and mature leaves have abundant auxin, which maintains an auxin gradient in the AZ and prevents an ethylene induction of abscission-related changes (abscission syndrome). CKs in the leaf prevent the premature induction of the senescence syndrome in the leaf. As the leaves age, the endogenous concentrations of both IAA and CKs drop below a threshold, allowing ethylene-induced senescence and abscission syndromes to be expressed.

10. OTHER HORMONES

Although most work on the abscission of leaves, flowers, and fruits highlights the role of ethylene as a natural regulator of abscission, many reports implicate ABA or jasmonates in the abscission process. As mentioned earlier, abscission of unpollinated ovaries or flowers is a common phenomenon. In a study involving cocoa flowers, it was shown that abscission of such flowers was correlated with an increase in ABA content. Also, the application of the carotenoid synthesis inhibitor, fluridone, which has the effect of lowering the endogenous ABA content (see Chapter 10), completely abolished both the formation of an AZ and the senescence and abscission of flowers. In contrast, the use of aminoethoxyvinylglycine, an ethylene synthesis inhibitor, delayed but did not prevent abscission.

ABA may also play an indirect role in the abscission of leaves caused by water deficit. For instance, in citrus plants exposed to water stress, a rise in ABA content in the roots is correlated with an increase in ACC content. The latter compound, an immediate precursor of ethylene, is transported to the shoot, resulting in an increase in ethylene concentration in the leaves and their abscission.

Jasmonates have also been implicated in abscission. They probably are involved in the synthesis of some defense-related proteins, but whether they serve to induce genes for wall hydrolases is not known.

11. SECTION SUMMARY

Abscission of organs occurs widely in plants. Organs that have served their function, are ripe, or are no longer wanted are shed. It is a controlled process that, in most cases, is initiated during the development of the organ by the formation of an abscission zone. In the AZ, abscission actually is confined to cells in the separation layer. The separation is affected by the secretion of polygalacturonases and cellulases that are specific to abscising organs and dissolve the middle lamella and disrupt the hemicellulose network in the primary walls between two layers of cells. The newly exposed cell surfaces are protected from pathogenic attack by the synthesis of many defense-related proteins and from water loss by the formation of a periderm and plugging of lumens of broken xylem vessels and tracheids. Ethylene accelerates the rate of abscission in many plants by inducing the synthesis of wall hydrolases in the AZ. IAA moving basipetally from the leaf blade is thought to retard abscission by maintaining the AZ in an ethylene-insensitive state. It also inhibits the expression of genes encoding abscission-specific cellulases and PGs. As leaves age, the concentration of auxin drops below the threshold required, and the ethylene-induced abscission syndrome commences. Ethylene also induces the expression of at least some genes encoding pathogenesis-related proteins. Ethylene may not be the only hormone promoting abscission. Absciscic acid and jasmonates have also been reported to induce abscission in some cases, but data are still very fragmentary.

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3

Seed Food Reserves and Their Accumulation

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1. WHAT ARE RESERVE FOODS?

Seeds store large amounts of food reserves, proteins, lipids, and polysaccharides, which are utilized following germination for growth of the seedling before it becomes autotrophic. These reserves are also a very major component of human (and animal) diet; thus, a great deal of research has been done on their

chemistry and molecular biology. In more recent years, efforts have been devoted to improving the quality of seed proteins and lipids by genetic engineering. The subject of seed food reserves is vast. A brief introduction is provided here. The interested reader is referred to other major texts and reviews on specific topics given in the references.

2. DIFFERENT AMOUNTS AND TYPES OF RESERVE FOODS ARE ACCUMULATED

Species differ as to the amounts and types of food reserves they accumulate in their seeds. Some species store relatively little food reserves (relative to seed weight); these seeds must be in the soil near the surface so that they can become autotrophic very quickly after germination (e.g., dandelion, California poppy). In others, large amounts of food reserves are stored in the seed (e.g., cereals, legumes—domestication has enlarged these seeds much beyond the size of their wild-type relatives). The food reserves are synthesized *in situ* from photoassimilate translocated to the developing seed from the mother plant *via* the long-distance translocation pathway of the phloem.

Seeds of most species accumulate two or more types of reserves, although their proportions vary (Table A3-1) Oil seeds (e.g., canola, soybean, oil palm) have more lipids than proteins or carbohydrates, whereas cereals have more carbohydrates than proteins or lipids. Most legumes accumulate far more protein than cereals, but because the gross tonnage of world cereal production,

TABLE A3-1 Food Reserves and Their Sites of Storage in Some Important Crop Species^a

	Average percent composition			Major storage organ
	Protein	Oil	Carbohydrate ^b	
Cereals				
Barley	12	3 ^c	76	Endosperm
Dent corn (maize)	10	5	80	Endosperm
Oats	13	8	66	Endosperm
Rye	12	2	76	Endosperm
Wheat	12	2	75	Endosperm
Legumes				
Broad bean	23	1	56	Cotyledons
Garden pea	25	6	52	Cotyledons
Peanut	31	48	12	Cotyledons
Soybean	37	17	26	Cotyledons
Other				
Castor bean	18	64	Negligible	Endosperm
Oil palm	9	49	28	Endosperm
Rapeseed	21	48	19	Cotyledons
(canola)				
Pine	35	48	6	Megagametophyte

^aFrom Bewley and Black (1994) with kind permission from Kluwer.^bMainly starch.^cIn cereals, oils are stored within the scutellum, an embryonic tissue.

about 2 billion tons, is about 12 times that of legumes (World Food Organization figures for 1990), cereals are still the most important source of vegetable proteins. In most conifers, proteins and lipids predominate but some carbohydrates also occur.

In seeds where the endosperm is the principal site of reserve food storage (e.g., cereals), smaller and sometimes qualitatively different types of reserves are stored in the embryo. Likewise, in seeds where the cotyledons or the megagametophyte is the principal site of storage, smaller amounts also occur in the root-shoot axis or in the embryo, respectively.

2.1. Intracellular Sites of Storage

Irrespective of the tissue in which they occur, proteins are stored in discrete membrane-bound structures called protein bodies or protein storage vacuoles (PSVs), which either arise from preexisting vacuoles or from the endoplasmic reticulum (ER) (Fig. A3-1). Different types of proteins may be stored in the same PSV, and some protein fractions may occur in a crystalline form, known as crystalloids, which are embedded in an amorphous matrix of other proteins. Lipids also occur

in the cytoplasm as discrete spherical bodies, surrounded by a membrane. Starch is the major carbohydrate reserve; it is deposited in the form of granules inside plastids that are specialized for starch accumulation (amyloplasts). In many seeds, large amounts of hemicelluloses are deposited in cell walls of the endosperm and serve as carbohydrate reserves.

3. STORAGE OF MINERALS

In addition to food reserves, seeds also accumulate many essential minerals. These minerals, potassium, calcium, and magnesium, also smaller amounts of iron, copper, and manganese, often occur as mixed salts of *myo*-inositol hexaphosphoric acid (**phytic acid**). The salt is called **phytin**. Phytin is deposited inside protein bodies, sometimes dispersed or aggregated in structures known as **globoids** (see Fig. A3-1). The synthesis of phytin, as well as its accumulation inside PSVs, is not well understood, but likely phytin is secreted into ER vesicles and the vesicles then fuse with the PSVs.

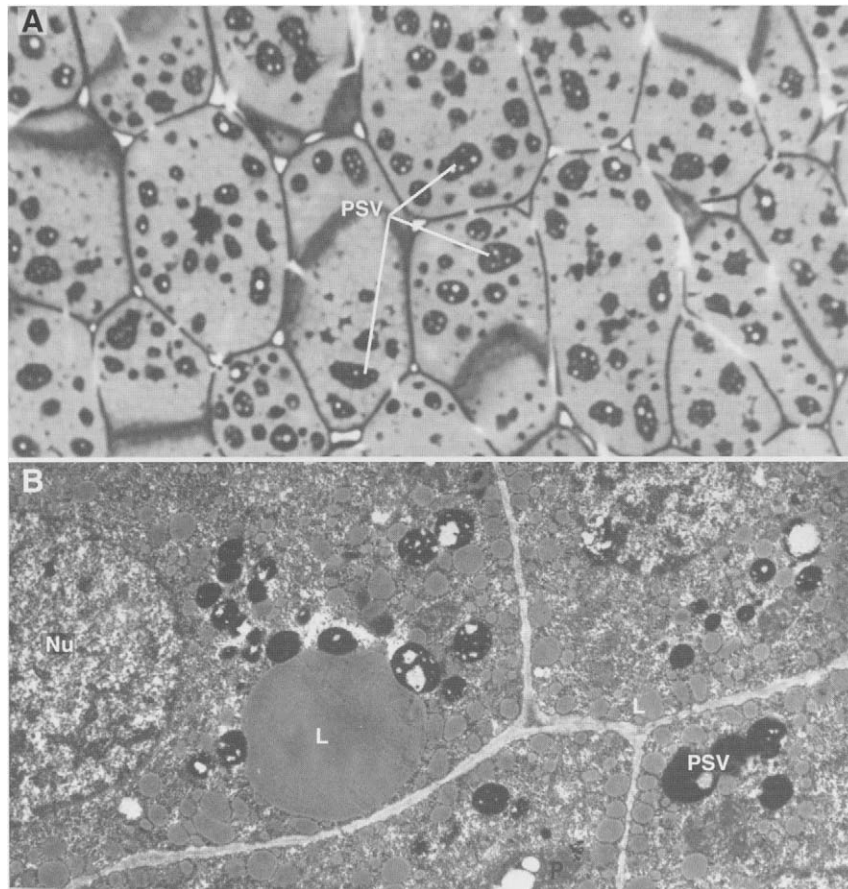


FIGURE A3-1 Light (A) and electron (B) micrographs of an ungerminated lettuce (*Lactuca sativa*) embryo showing protein storage vacuoles (PSV) and lipid droplets (L). PSVs show light structures known as globoids, which consist of phytin. Lettuce seeds do not deposit starch grains. Nu, nucleus.

4. TRANSLOCATION OF PHOTOASSIMILATE TO THE ENDOSPERM AND THE EMBRYO

The developing seeds, like fleshy fruits, are strong sinks for photoassimilate. In addition, in some plants, carbon and nitrogen reserves in vegetative parts are mobilized and rerouted to developing seeds and fruits (see Chapter 20). Typically, the symplastic translocation of photoassimilate *via* the phloem from the mother plant to the seed stops at the funiculus or, at the farthest, the seed coat—there are no vascular connections between the endosperm or the embryo on the one hand and the seed coat on the other. Moreover, irrespective of whether the endosperm is resorbed or not, the embryo (root–shoot axis and cotyledons), for the most part, lies free in the endosperm (Fig. A3-2), which means that the final translocation of photoassimilate to the embryo occurs apoplastically. The growing embryo is bathed in a nutritive solution and absorbs its nutrients directly

from the “periembryonic” space. [A “seed cup technique” where the embryo is removed surgically, leaving an empty cup of seed coat, still connected via funiculus to the mother plant, has been used to study such translocation. The technique, however, is best used with certain types of seeds, such as legume seeds.] Specialized parenchyma cells, called transfer cells, occur in the epidermal layer of the cotyledons, or endosperm, and the innermost cell layer of the seed coat in several dicot and cereal species. These cells have wall invaginations and an enhanced surface area of plasma membrane and facilitate the secretion or uptake of solutes.

The photoassimilate imported into the seed consists mainly of sucrose and amino acids, particularly glutamine and asparagine. An invertase in the periembryonic space hydrolyzes the maternal sucrose into glucose and fructose, which are taken up by the endosperm/embryo and reconverted to sucrose for further symplastic translocation in the embryo. The uptake of amino acids and sugars (sucrose and hexoses) into the apoplastic space and from that space into epidermal cells of embryo is

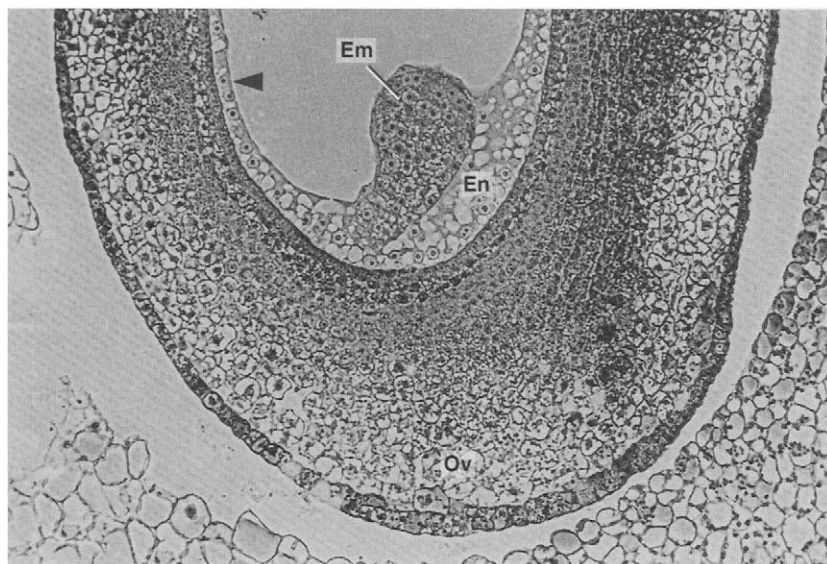


FIGURE A3-2 A young ovule (Ov) taken from a 0.4-cm-long pod of peanut (*Arachis hypogea*). In peanut, the endosperm degenerates and food is stored in the cotyledons and root-shoot axis. At this stage, the globular embryo (Em) is embedded in the endosperm (En), which is partly coenocytic (arrowhead) next to the embryo sac wall. Later, the degenerating endosperm may also provide nutrients to the embryo, which are taken up apoplastically. $\times 115$. From Zamski (1995).

facilitated by carrier proteins, or transporters, which are coupled to H^+ -ATPases (see Box 13-1, Chapter 13). Specific transporters for amino acids, sucrose, or hexoses are known and their cDNAs have been cloned

from several plants (e.g., fava bean, *Arabidopsis*, garden pea). Moreover, the transporter proteins and their transcripts have been localized in epidermal cells of cotyledons that show transfer cell morphology (Fig. A3-3).

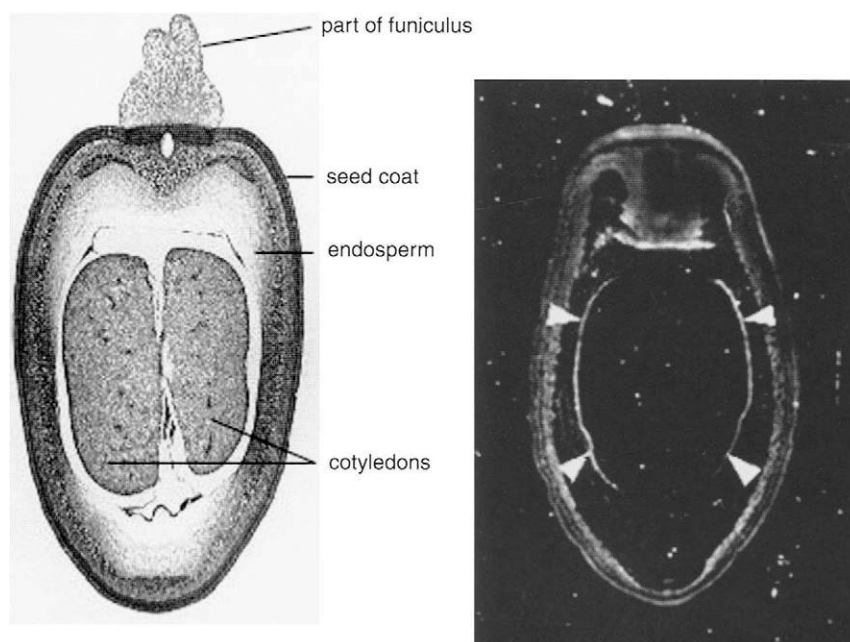


FIGURE A3-3 Tangential section of a developing fava bean (*Vicia faba*) seed. The bright-field image (left) shows part of the funiculus, the seed coat, the endosperm, and the two cotyledons (embryo). Vascular bundles are cut at various places in the seed coat and the cotyledons. The cotyledonary epidermis is the site of transfer cells. The dark-field image (right) shows strong labeling of the epidermal layer after *in situ* hybridization with a sucrose transporter cDNA (arrowheads). Courtesy of Hans Weber Institut für Pflanzen-genetik und Kulturpflanzenforschung, Gatersleben, Germany.

Amino acids and sugars taken up by the embryo/endosperm are used for the synthesis of building blocks for different types of food reserves (Fig. A3-4).

5. CARBOHYDRATE RESERVES

5.1. Starch

Starch is the major carbohydrate reserve in seeds of most plants and is deposited in the form of starch granules inside amyloplasts, which, in late stages of starch deposition, may be so full as to burst and release the granules in the cell cytoplasm. In cereals, starch is accumulated mainly in the cells of starchy endosperm, which die at seed maturity, and the starch grains occur tightly adpressed against the protein bodies. In contrast, in dicots such as pea and bean, starch is accumulated in the cells of the embryo—the cotyledons and root–shoot axis—which continue to live. This difference between starch storage in cereals vs legumes has implications in the hormone-mediated mobilization of food reserves in the two groups of plants (see Chapter 19, Section II).

5.1.1. Structure and Synthesis of Starch

Starch is a mixture of two structurally different glucans, amylose and amylopectin, which occur in

varying proportions in starch from different sources; amylose about 20–30% and amylopectin about 70–80%. Amylose consists of linear chains of 300–400 α -D-glucopyranosyl residues linked by α -(1–4) bonds. Amylopectin has the same backbone, but is a branched polymer consisting of many amylose chains linked by α -(1–6) bonds (Fig. A3-5); it is also much larger (100- to 1000-fold larger). These polymers are deposited at nucleation sites in amyloplasts and lead to the formation of starch granules, which vary in size from a few micrometers to as much as 100 μ m in diameter; granules of different sizes may occur in the same cell.

Three major enzymes participate in starch synthesis (Fig. A3-6). ADPglucose pyrophosphorylase (ADPGlc PPase) catalyzes the synthesis of ADPglucose (ADPGlc) from glucose-1-phosphate (Glc-1-P) and ATP with release of the pyrophosphate. A second enzyme, starch synthase (SS), transfers the glucosyl unit of ADPGlc to the nonreducing end of a preexisting α -1,4-glucan primer. A third enzyme, the starch-branching enzyme (SBE), hydrolyzes an α -1,4 bond and attaches the released α -1,4 glucan segments to the same or another glucose chain by an α -1,6 linkage. The reaction catalyzed by SBE creates branch points and new nonreducing ends for further elongation of the α -1,4 glucan branches.

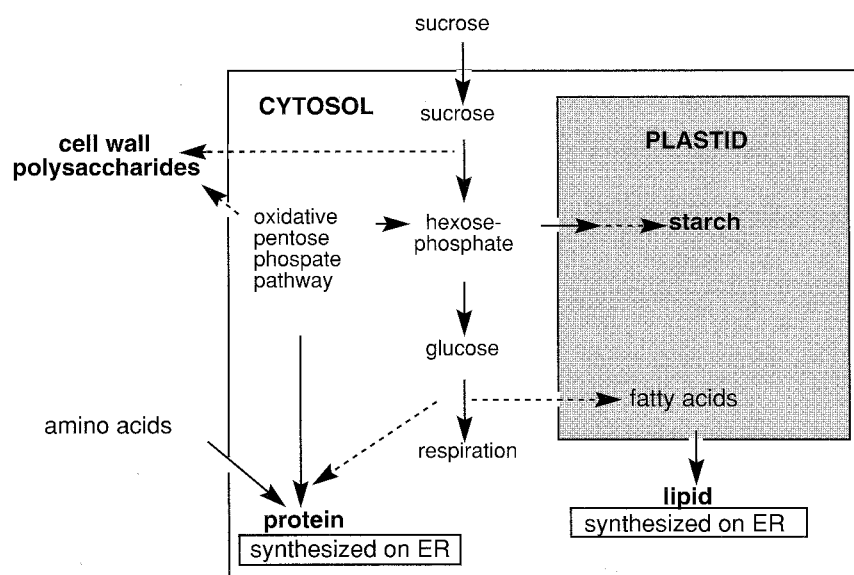


FIGURE A3-4 Generalized relationship between sucrose metabolism and synthesis of storage products and cell wall polysaccharides (highlighted in bold letters). A nonspecific term “plastid” is used to indicate that starch and fatty acids are synthesized in that organelle. Hexose-phosphates imported into the plastids in storage organs include glucose-6-phosphate, glucose-1-phosphate, and ADPGlc, not a triose phosphate, as is typical in photosynthetic cells. Adapted from Smith and Denyer (1992).

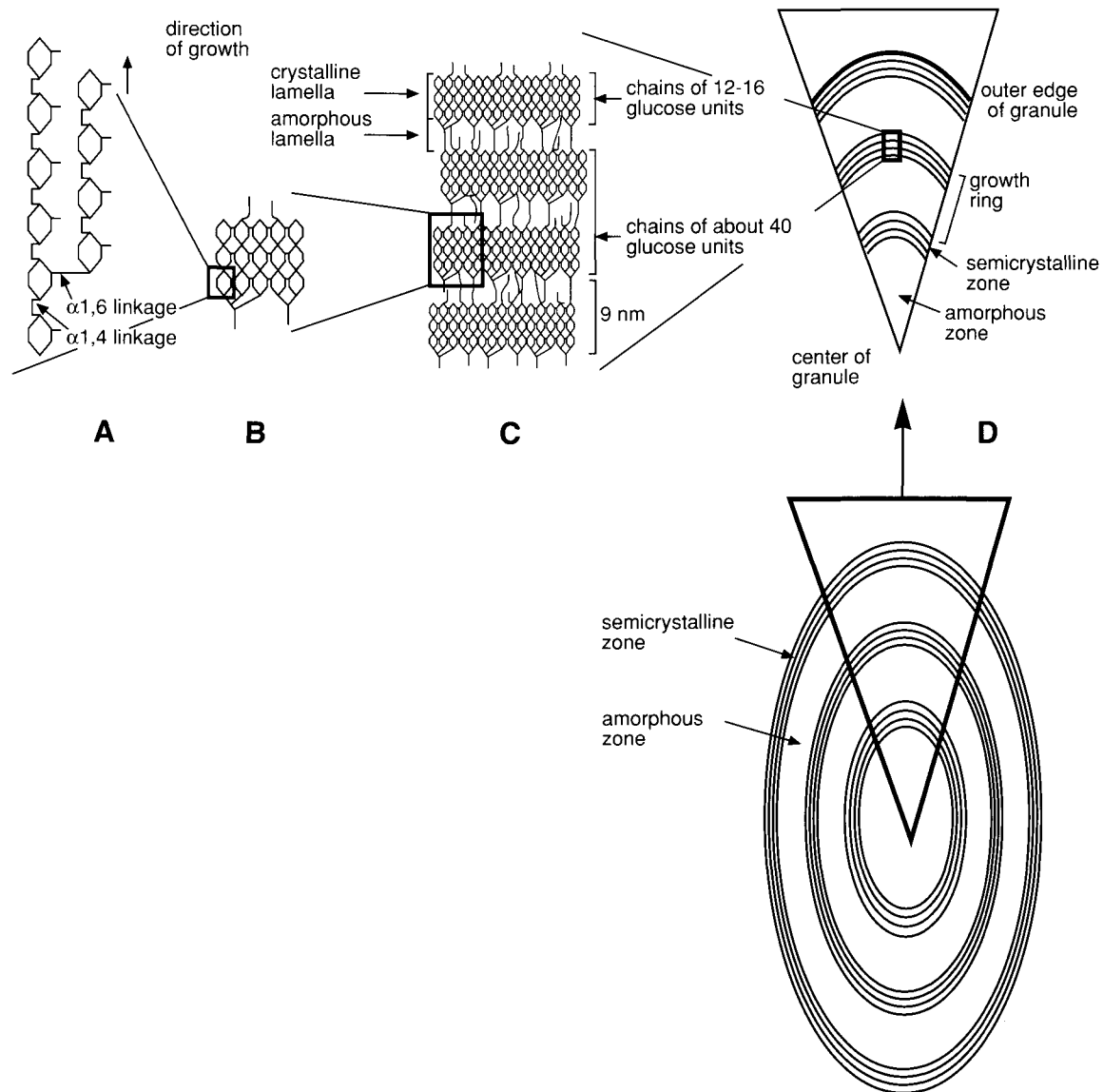


FIGURE A3-5 Schematic representation of levels of organization within the starch granule. Boxes in B, C, and D represent the area occupied by the structure in the preceding panel. (A) Two branches of an amylopectin molecule, each consisting of $\alpha(1 \rightarrow 4)$ -linked glucan chains (amylose) attached by an $\alpha(1 \rightarrow 6)$ linkage. (B) A cluster within an amylopectin molecule showing the association of adjacent branches. (C) Clusters are arranged to form alternating crystalline and amorphous lamellae. Crystalline lamellae are more closely packed and represent a more ordered arrangement of amylose and amylopectin chains than amorphous lamellae; the two together form the semicrystalline zone. (D) Slice through a starch granule showing alternating zones of semicrystalline and amorphous zones. From Smith (1999) with permission from Elsevier Science.

ADPGlc PPases in higher plants are large tetrameric holoproteins with two large (54–60 kDa each) and two small subunits (51–55 kDa each). The large and small subunits are encoded by multigene families, and individual isoforms show tissue specificity in expression; some are specifically expressed in seed tissue and others in leaf, root, tuber, etc. Mutations affecting the two subunits are known (Table A3-2) and results in

much reduced starch content. For instance, one of the lines of sweet corn is given by the *shrunk2* mutation, less starch is formed, and more sucrose precursor builds up.

The SS occurs in two forms: one bound to the starch granules (granule-bound SS or GBSS) and the other as a soluble protein (SSS). Each has several isoforms. Both soluble and granule-bound enzymes play a role in

TABLE A3-2 Starch Synthesis Mutants

Enzyme type	Mutant	Component affected	Plant
ADPGlcPPase	<i>shrunk2</i>	Large subunit	Maize
	<i>brittle 2</i>	Small subunit	Maize
	<i>rugosub</i>	Large subunit	Pea
SS	<i>waxy</i>	One of the GBSS	Maize, rice, potato tuber
SBE	<i>sbe1</i> and <i>sbe2</i>	Two SBEs (in family A)	Maize
	<i>rugosus</i>	SBE (family A)	Pea

^aCompiled from Martin and Smith (1995) and Gao *et al.* (1996).

starch formation. The GBSS produces more amylose, whereas the soluble forms are thought to be more involved in the production of amylopectin. In the *waxy* (*wx*) mutant of cereals, there is virtually no production of amylose. The protein encoded by the wild-type *WAXY* gene in maize occurs associated with starch grains, and this protein is missing from the *wx* mutants, which suggests that the *WX* protein is a GBSS.

The SBE, the key enzyme for production of amylopectin, has been characterized from many plants (e.g., pea embryo, maize endosperm, potato tuber, leaf tissues). It also occurs in multiple isoforms, which are placed in two families: A and B. Members of the two families differ from each other in molecular size, immunological reactivity, substrate preference, and developmental expression. Mutants defective in SBEs belonging to family A show reduced starch content, but more specifically much less amylopectin (~30% of that in WT). They produce more amylose than normal and also a highly branched but soluble glucan polymer. They also retain much more sucrose, which alters the osmotic properties of the developing seed. At maturity, with desiccation, these seeds show many abnormalities, including fissures, and a wrinkled appearance. Mendel's *wrinkled* pea mutant (*rugosus* in Table A3-2) had a defective SBE. Wrinkled peas are sweeter because there is more sucrose and less starch, which is why they are used in the food industry. Mutants belonging to family B are still unknown, probably because they do not produce a distinctive seed phenotype and have gone undetected.

5.2. Hemicelluloses in Cell Walls

Hemicelluloses, such as mannans, galactomannans, glucomannans, and arabinoxylans, are often deposited as reserve foods in cell walls of storage tissues, such as endosperm or perisperm (Table A3-3). The walls can become very thick and occlude the lumen, resulting

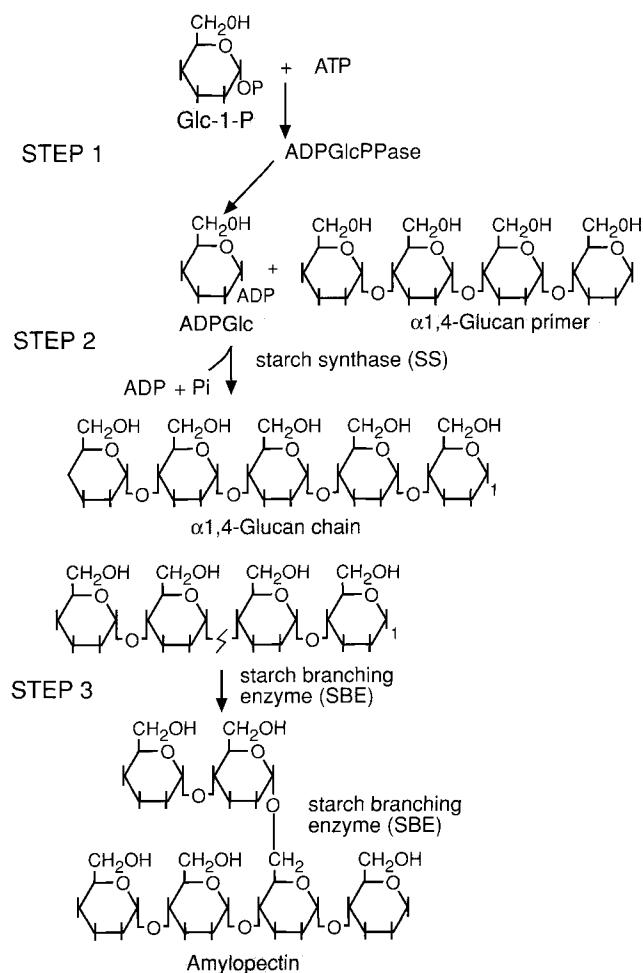


FIGURE A3-6 Steps in starch synthesis. Step 1: ADPGlc PPase catalyzes the formation of ADPGlc and inorganic pyrophosphate from Glc-1-phosphate and ATP. Step 2: Starch synthase (SS) adds glucose units from ADPGlc to the nonreducing end of a growing $\alpha(1 \rightarrow 4)$ -linked glucan chain by an $\alpha(1 \rightarrow 4)$ -linkage and releases ADP and Pi. Step 3: The starch-branching enzyme (SBE) cuts an $\alpha(1 \rightarrow 4)$ -linked glucan chain and forms an $\alpha(1 \rightarrow 6)$ -linkage between the reducing end of the cut chain and the C6 of another glucose residue in an $\alpha(1 \rightarrow 4)$ -linked chain, thus creating a branch.

TABLE A3-3 Hemicelluloses Deposited as Food Reserves in Cell Walls

Storage tissue	Family	Plant
Endosperm	Leguminosae	Fenugreek (<i>Trigonella foenum graecum</i>)
		Guar (<i>Gleditsia triacanthos</i>)
		Clover (<i>Trifolium incarnatum</i>)
		Carob (<i>Ceratonia siliqua</i>)
	Arecaceae	Date palm (<i>Phoenix dactylifera</i>)
Perisperm (remains of nucellus)	Cucurbitaceae	Ivory nut (<i>Phytalephas macrocarpa</i>)
		Muskmelon (<i>Cucumis melo</i>)

in dead cells at maturity (e.g., fenugreek, clover); in other cases, the cells remain living (e.g., date palm, Fig. A3-7). The texture of the storage tissue depends on the kinds of hemicellulose present. An excess of pure mannans often imparts a hard texture, whereas the presence of gluco- or galactomannans imparts a softer texture. In some plants, such as lettuce (*Lactuca sativa*) and tomato (*Lycopersicon esculentum*), the reserves in the endosperm get resorbed by the embryo early in development, but the endosperm walls at maturity have a high proportion of mannans and galactomannans, which may still be utilized as reserve food after germination.

5.3. Sugars

Free sugars (such as, sucrose, raffinose, stachyose) are always present in seeds. Their role, if any, as protectants of cell structure in case of water stress during early seed development is unknown, but they are essential protectants against desiccation damage during seed maturation. They also serve as the first source of carbohydrates for respiration and metabolism during germination before mobilization of reserve foods. In some seeds, they may account for up to 11% of the dry weight of the seed (e.g., sugar maple). Free hexoses are not deposited.

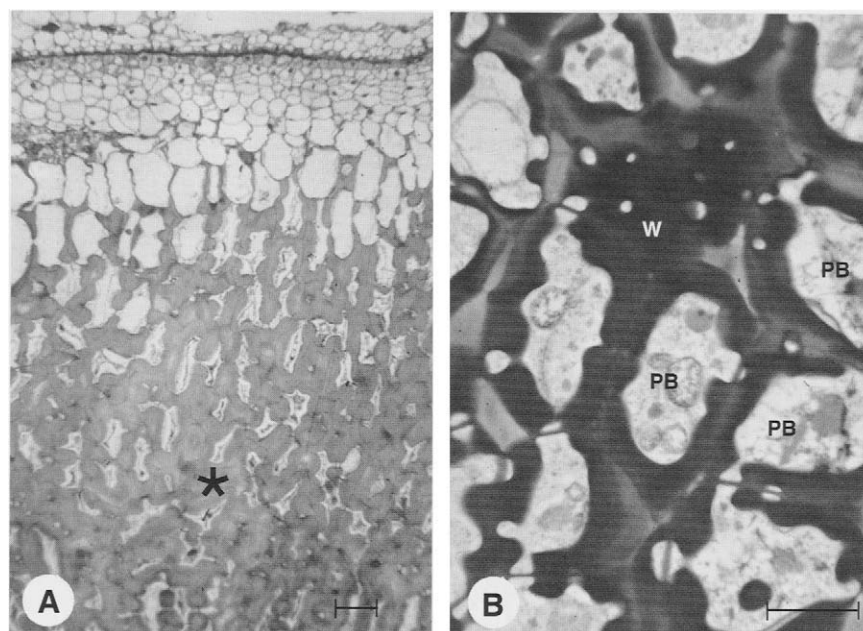


FIGURE A3-7 Cell walls of date palm (*Phoenix dactylifera*) endosperm. (A) Endosperm at 11 weeks after pollination. (B) Enlarged view of cells from central endosperm (*). Note thick striated cell walls. There is no starch, but storage protein bodies are still deposited. PB, protein bodies; W, cell wall. Scale bars: 50 μ m. From DeMason *et al.* (1989).

6. LIPIDS

6.1. Oil Bodies and Oleosins

Seed storage lipids are mainly oils, i.e., liquid above 20°C, and almost always triacylglycerols (TAGs), i.e., esters of glycerol and fatty acids (see Box 12-2 in Chapter 12). One exception is jojoba (*Simmondsia chinensis* L.) seed. It stores liquid wax formed by the acylation of fatty acids to long-chain alcohols.

Lipids are stored in seeds in discrete membrane-bound structures known as lipid bodies (also known as oleosomes or spherosomes). The membrane surrounding the lipid bodies is not the usual bilayer membrane, but a half-membrane, with the nonpolar fatty acid chains toward the body of the lipid and the polar heads facing the cytoplasm. Lipid bodies in desiccation-tolerant seeds show the presence of a protein,

called **oleosin**, which occurs in the monolayer of phospholipids (Fig. A3-8). Oleosins (M_r s 16–25 kDa) are unique to oil bodies and are unusual proteins. Their N and C-termini are variable, but a central stretch of 70–80 amino acids is conserved and highly hydrophobic. They are thought to be shaped like thumbtacks with the hydrophobic part folded back and forming the pin, which is embedded in the body of the lipid, whereas N and C termini forming the “head” domains are amphipathic. The exact function of oleosins is unknown, but because they are usually absent from tropical seeds that do not desiccate, it is thought that they stabilize the oil bodies and keep them from coalescing into a larger diffuse mass on rehydration of dry seeds. Discrete oil bodies may provide a higher surface area to volume ratio than a diffuse mass and thus permit a more efficient mobilization of triacylglycerols following seed germination.

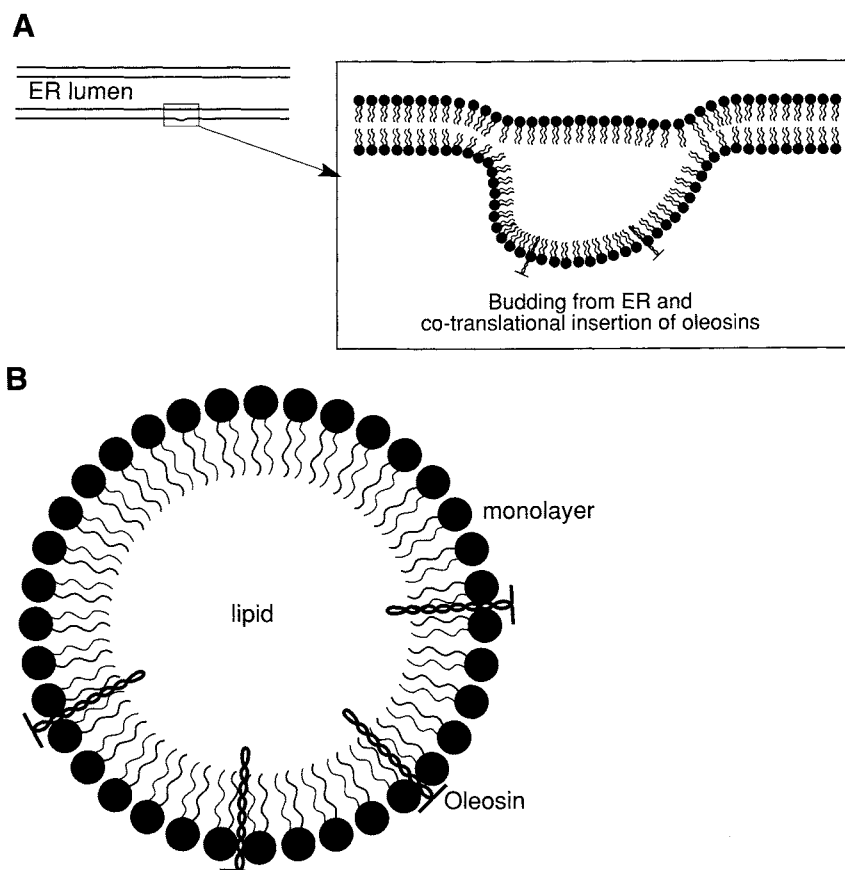


FIGURE A3-8 Postulated origin of lipid body from the endoplasmic reticulum (ER). (A) Lipid bodies are synthesized on the ER membrane and deposited between the bilayer of a membrane. Adapted from Napier *et al.* (1996) with kind permission from Kluwer. (B) After a certain size has been reached, they are extruded out with the half-membrane surrounding the lipid body. Oleosin molecules are concomitantly intercalated between the phospholipids in the monolayer.

6.2. Fatty Acid Composition Varies Widely in Seed Oils

Fatty acids differ from each other in the number of C atoms, from 12-C to 24-C, and in the number of double bonds in the chain, from none to one, two, or three. Seed oils in different species vary widely in the proportion of different fatty acids, although 18-C unsaturated fatty acids generally predominate. Eight fatty acids are most common (five are listed by name in Table A3-4). In marked contrast to membrane glycerolipids where fatty acid chain lengths are highly regulated because of steric considerations, seed lipids show the greatest diversity of fatty acids among organisms. More than 300 different fatty acids are known, many with unusual fatty acids with chain lengths from 8-C to > 22-C, and some with an unusual number of double bonds and oxidations or epoxidations. The reason for this diversity is not understood.

6.3. Synthesis of Oil Bodies

The esterification of glycerol with fatty acids occurs in the cytoplasm, and newly synthesized TAGs accumulate in discrete organelles, the oil bodies. The size of the oil bodies is species dependent and ranges between 0.6 and 2.0 μm in diameter; new ones being

formed as more lipid is synthesized. Lipid synthesis occurs on specific domains of tubular (smooth) ER where the enzymes for TAG synthesis are located. The newly synthesized TAG molecules accumulate between the two leaflets of a bilayer in the ER membrane, and the lipid droplet, after a certain size has been reached, is budded off carrying the single leaflet with it (see Fig. A3-8). Oleosins are synthesized concomitantly and inserted in the monolayer. It is further possible that the insertion of oleosins specifies the site for TAG synthesis and assembly.

7. PROTEINS

Storage proteins are divided into four classes based on their solubilities: albumins, globulins, prolamins, and glutelins (Table A3-5). This classification provides a useful approach to the extraction of proteins, although it should be recognized that the solubilization of proteins is affected by their tertiary and quaternary structures and by solvents. In more recent years, glutelins are regarded as a subgroup of prolamins with interchain disulfide bonds and, hence, different solubility characteristics (see Section 7.2).

Seed storage proteins are distinct from proteins in vegetative parts. They are specifically produced

TABLE A3-4 Composition of Major World Oil Crops and Some Oilseeds of Interest

Oilseed	Typical fatty acid composition (% by weight)						Other
	<16:0	Palmitic 16:0	Stearic 18:0	Oleic 18:1	Linoleic 18:2	Linolenic 18:3	
Oil crops							
Soybean	—	11	3	22	55	8	1
Palm	—	47	4	38	10	—	—
Rapeseed (canola)	—	4	2	59	23	8	3
Sunflower	—	7	3	14	75	—	—
Cotton	1	25	3	17	53	—	—
Peanut	—	11	2	51	31	—	2
Coconut	80		4	5	3	—	—
Castor bean	—	1	1	3	4	Traces	90
Oilseeds of interest							
<i>Cuphea</i>	96	1	Traces	1	2	Traces	—
Coriander	<1	5	Traces	6	13	Traces	75
Meadowfoam	—	<1	<1	<1	1	<1	96

^aFrom Miquel and Browse (1995).

TABLE A3-5 Classification of Plant Storage Proteins^a

Protein type	Soluble in
Albumins	Water or dilute buffers at neutral pH
Globulins	Salt solutions
Prolamins	Alcohols (70–90%)
Glutelins	Dilute acids or alkalis

^aAfter Osborne (1924).

in seed tissues, not in vegetative parts of the plant, although their genes can be expressed ectopically in vegetative tissues. Various lectins, toxins, and inhibitors are often included with storage proteins, but LEA proteins, other stress-related proteins, and proteins associated directly or indirectly with seed maturation or dormancy (if any) are excluded. This separation is not easy because many of these proteins are synthesized at about the same time in seed development—mid- to late embryogenesis—and, in some cases, under the same inducing stimulus, abscisic acid.

7.1. Types and Occurrence of Storage Proteins

A globulin (or prolamin) fraction from a seed usually consists of not one, but a number of different proteins, which are further separated according to their molecular weights by electrophoresis on a gel or sedimentation coefficients in a sucrose density gradient.

Dicot and conifer seeds typically store globulins. Globulins are separated into two major types, **legumins** and **vicilins**, which have sedimentation (S) coefficients of 11–12S and 7S, respectively (the actual S values vary depending on species and extraction conditions). 2S proteins are also found in dicot seeds and include both globulins and albumins. The proportions of legumins, vicilins, and albumins vary considerably among different dicot species. In some, nearly 50% of the storage protein is composed of legumins [e.g., pea (*Pisum sativum*), soybean (*Glycine max*)], whereas in others, vicilins predominate [e.g., fava bean (*Vigna radiata*), bean (*Phaseolus vulgaris*)]. In some species, one or the other kind may be produced exclusively.

Cereals have prolamins in addition to globulins and albumins. Prolamins are the major proteins in the members of Triticeae (wheat, barley, rye) and Panicoideae (maize, sorghum, millet). In wheat, substantial

amounts of prolamins with interchain disulfide bonds occur, which give “gluten” the characteristic viscoelastic properties of wheat bread dough. In contrast, in oat and rice (members of Aveneae and Oryzeae, respectively), 11S globulins are the major storage proteins (in rice, it is sometimes referred to as a glutelin). Cereals, like dicots, store a variety of 2S proteins.

Major seed storage proteins are often given trivial names after the genus or species, sometimes family, in which they occur, e.g., zein in maize (*Zea mays*), hordein in barley (*Hordeum vulgare*), helianthinin in sunflower (*Helianthus annuus*), and napin and cruciferin in rapeseed (*Brassica napus* and family Cruciferae). Genes encoding these proteins are similarly named.

Lectins are a heterogeneous group of defense-related proteins. They are present in seeds as well as in vegetative tissues, such as stems and leaves, and are covered in greater detail in Chapter 20. Examples of lectins in seeds include phytohemagglutinin (PHA) in *Phaseolus vulgaris*, concanavalin A (Con A) in jack bean (*Canavalia ensiformis*), and wheat germ agglutinin (WGA) in wheat. Some seed lectins are highly toxic. For example, ricin D from castor bean and abrin from *Abrus precatorius* (rosary pea) are both ribosome-inactivating proteins.

Individual protein storage vacuoles in dicots usually accumulate several different types of proteins, although in some cases only one type, e.g., only albumin, vicilin, or legumin, may be stored. In cereals, likewise, PSVs may contain only prolamin (e.g., endosperm of maize or sorghum) or contain prolamins and 11–12S globulins (e.g., endosperm of oat, wheat, barley). Also, PSVs in different parts of the seed, i.e., the embryo or the endosperm, may accumulate different types of proteins, i.e., there may be tissue specificity in protein storage. For example, in cereals, the starchy endosperm typically stores prolamins and/or 11–12 S legumins, whereas the embryo and aleurone tissue store 7S vicilins, although in proportionally small amounts.

Since seed storage proteins occur in abundance, their genes were among the first plant genes to be studied. Nearly all the major storage protein genes from dicots and cereals have been cloned and characterized as to their structure, regulatory *cis* sequences, and evolutionary relationships. A consideration of those topics is beyond the scope of this book. In the following, some important features about the structure, processing, and storage of seed proteins are highlighted, but before doing so, some general features of protein processing and targeting are outlined in Box A3-1.

BOX A3-1 PROCESSING AND TARGETING OF PROTEINS

A METABOLICALLY ACTIVE PLANT cell produces hundreds of different proteins each day, which are processed, sorted, and targeted to different locations within the cell. These processes occur in the endomembrane system of the cell, which typically consists of the nuclear envelope, the ER, the Golgi network, and vacuole(s). This system is highly complex and shows not only morphological, but also biochemical compartmentation. For instance, the ER is often distinguishable into rough and smooth ER (i.e., RER or SER), and the RER may further show functional specialization in that certain parts are devoted to one type of activity, whereas other parts are devoted to other activities. Golgi bodies typically have a stack of three to eight flattened cisternae, with a distinct polarity, the forming or *cis* face, one or more cisternae in the middle, and a maturing or *trans* face where vesicles from Golgi bud off. However, the Golgi are not isolated structures, but with their membranes and vesicles form the Golgi network, which interfaces on the *cis* side with the ER and on the *trans* side with the vacuoles (Fig. A3-9).

Proteins are synthesized on the rough ER. Those that are processed are inserted cotranslationally into the ER. Such insertion requires the presence of a signal peptide at the N terminus, which is recognized by a specific receptor (docking protein) on the ER membrane. Signal peptides vary in length from 16 to 32 amino acids; they are not specific to a polypeptide—they can be recognized by docking proteins in heterologous species, but they are essential for entry into the ER. After the entry, the signal peptide is cleaved by an endopeptidase located on the inner side of the ER membrane.

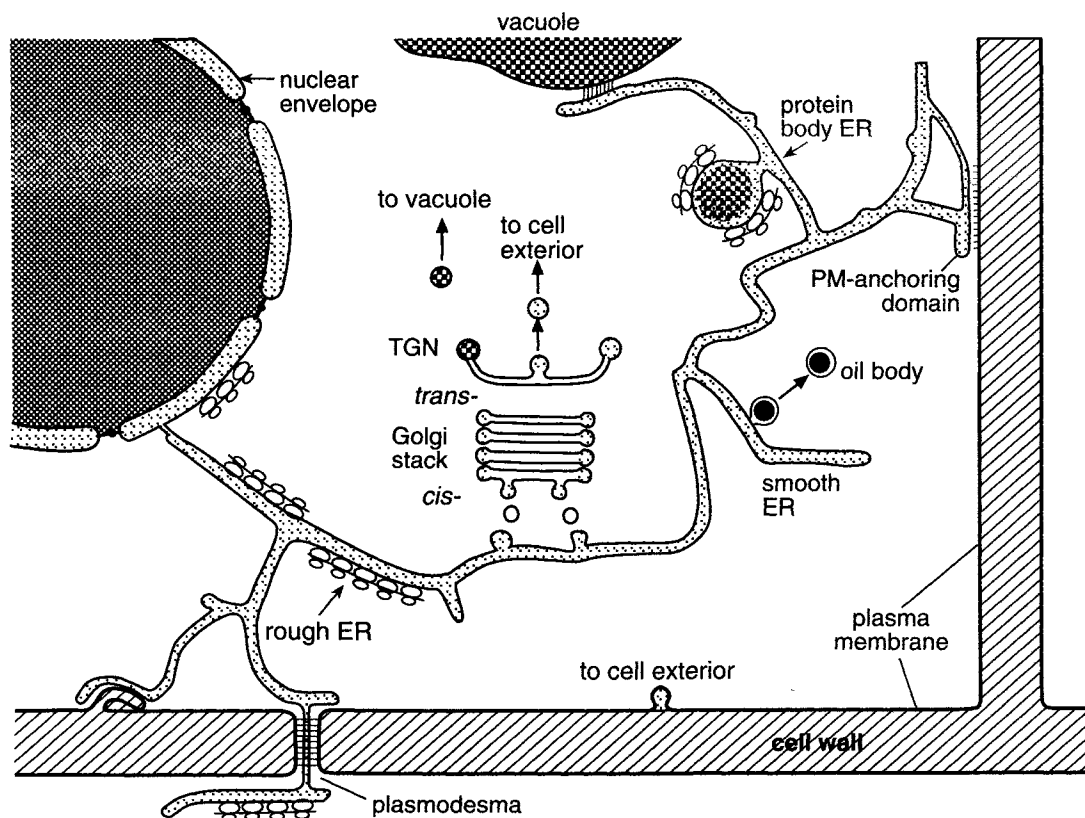


FIGURE A3-9 Endomembrane system in a plant cell. The endomembrane system consists of an interconnected network of the endoplasmic reticulum (ER), the nuclear envelope, Golgi bodies with their *cis*- and *trans*-Golgi vesicles, and vacuolar membranes. The ER is distinguished morphologically into rough or cisternal ER (RER) and smooth or tubular ER (SER), but functionally it is distinguished into many more domains: some devoted to protein synthesis and assembly, some to lipid synthesis and accumulation, and some to biogenesis of vacuoles. Still other domains connect it to mitochondria and plasma membrane. Modified with permission from Staehelin (1997), © Blackwell Science Ltd.

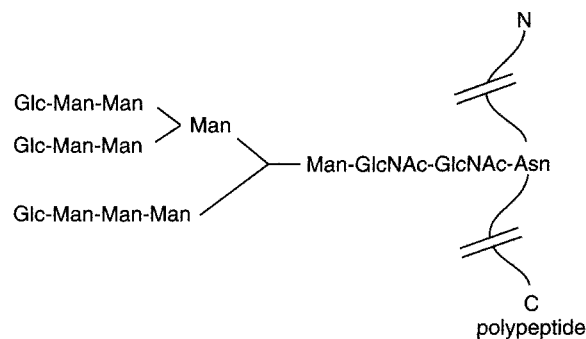


FIGURE A3-10 Glycosylation of a polypeptide in the ER. An oligosaccharide, consisting of $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$, is shown attached to an Asn residue in the polypeptide via an *N*-acetyl glucosamine. Following attachment, the glucose and some mannose residues may be trimmed to give a shorter mannose-rich chain. Adapted from Vitale and Bollini (1995).

Inside the ER, polypeptides undergo several types of modifications catalyzed by ER-resident enzymes. Glycosylation of proteins is common. It consists of attachment of one or more short oligosaccharide chains, typically composed of glucose, mannose, and *N*-acetyl glucosamine residues. The attachment is catalyzed by an oligosaccharyl transferase and occurs at specific Asn residues in the polypeptide chain (Fig. A3-10). After the attachment, some glucose and mannose residues are cleaved by specific glucosidases and an α -mannosidase to give shorter mannose-rich side chains.

Polypeptides are folded in specific conformations while within the ER. Such folding, in many cases, requires cross-linking by intra- or interchain disulfide bonds between specific cysteine residues. The formation of these bonds is catalyzed by the enzyme protein disulfide isomerase (PDI). An immunoglobulin-binding protein (BiP) is thought to play a role in ensuring that proteins transported *via* the *trans*-Golgi network are folded properly and assembled into transport-competent forms. Those that are folded incorrectly are removed from the transport pathway and are targeted for proteolytic digestion.

Many of these ER resident proteins, such as BiP and PDI, carry a C terminus tetrapeptide sequence, KDEL or HDEL. The presence of this sequence in a protein earmarks it as an ER resident protein, but not all polypeptides that reside in the ER for a short or long time have ER retention signals. How the KDEL or HDEL sequence allows retention of the protein within the ER is not fully understood.

Sorting of proteins for transit to various locations occurs in the Golgi network. The transit of secretion vesicles to the plasma membrane and exocytosis requires no signals—that is considered the default pathway. Transport to the nucleus, or organelles such as chloroplasts and mitochondria, or to vacuoles requires the presence of specific signals, strings of amino acids that act as address codes, and which are embedded within the amino acid sequence of the processed polypeptides (Fig. A3-11). The address codes are recognized by the Golgi vesicles. Golgi vesicles also carry specific signals, which, in turn, are recognized by the membrane of the target organelle. Specific proteins, called “syntaxins” (on the organelle membrane), are thought to aid in recognition. Further processing of the polypeptides, including cleavage of the propeptide, occurs in the destination organelle.

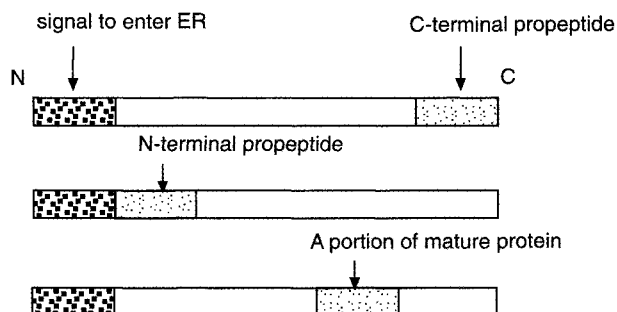


FIGURE A3-11 Specific amino acid sequences target polypeptides to specific intracellular destinations. The targeting sequence (the propeptide) in different proteins occurs near the N- or the C-terminal or in between. From Chrispeels and Raikhel (1992) with permission from Elsevier Science.

7.2. Structure, Processing, and Storage of Seed Protein Reserves

Storage proteins are typically oligomeric, i.e., they have several subunits; each subunit, in turn, may have more than one polypeptide chain. The whole, mature protein, the **holoprotein**, therefore, is a complex structure.

7.2.1. Dicots

Legumin holoproteins are hexameric, i.e., composed of six nearly identical subunits, each with a molecular mass of ~ 50 – 60 kDa. Each subunit, in turn, is composed of two polypeptide chains; α and β . The larger α chain is hydrophilic and has a weakly acidic pI; the smaller β chain is more hydrophobic and has a strongly basic pI. The two chains of a subunit are encoded by a single gene. The primary translation product, the protomer or the nascent polypeptide, carries the two putative chains, linked by a peptide bond. The nascent polypeptide is inserted cotranslationally into the ER, where the signal peptide is cleaved off and a disulfide bridge is formed between the two putative chains, giving rise to “prolegumin” (Fig. A3-12A). With few exceptions, both chains are unglycosylated. Three such subunits assemble to form a trimer, which is the form in which prolegumin is transported to the storage vacuole. In the vacuole, further processing and “maturation” of polypeptides into holoproteins occur. The peptide bond between the two putative chains is cleaved, but because they already are bound by a disulfide bridge, the two chains remain paired. The cleavage occurs at a specific peptide bond in the pre-protein precursor (see Fig. A3-12B). Cleavage of the peptide link is the signal for two trimers to come together and form a hexamer. Propeptides embedded in the amino acid sequences, directing transfer of the protein to the vacuole, are also cleaved.

Vicilin holoproteins are trimers, but they are composed of two types of subunits; one type is large (70 kDa) and the other is small (~ 50 kDa). For example, β -conglycinin, the major 7S globulin from soybean (*G. max*), has three long chains of 70 kDa each, whereas phaseolin, the major 7S globulin from bean (*P. vulgaris*), consists of three identical chains of 50 kDa each. Vicilin proteins are often glycosylated, but lack disulfide bridges between the subunits because they lack cysteine. Both types of protomers are inserted cotranslationally into the ER and the signal peptide is cleaved off. The large subunits usually undergo no further cleavage (Fig. A3-12A). In contrast, the smaller subunits are polymorphic and may undergo no further change (e.g., phaseolin) or undergo fragmentation into still smaller subunits (e.g., vicilin from pea or fava bean).

The 2S storage proteins are a heterogeneous group and include both globulins and albumins (e.g., napin, the 2S globulin from *Brassica napus*; α -amylase inhibitor in cereal grains). These proteins are monomeric, i.e., they are derived from a single translation product or protomer, but the processing of the protomer is complicated. For example, in napin, the protomer carries a signal peptide, which is followed by a small propetide, then the putative short chain, and then the long chain (Fig. A3-12A). The signal peptide is cleaved off, and the remainder in the ER undergoes disulfide linkage between the parts that are going to form the small and the large chain. Final processing occurs in the storage vacuoles, where vacuolar processing enzymes cleave the propetide, followed by cleavage of the peptide link between the small and the long chain.

7.2.2. Cereals

Prolamin holoproteins are composed of several polypeptides (monomers), and the individual polypeptides may be products of separate genes (e.g., α , β , γ , and δ zeins in maize). In some prolamins, individual polypeptide chains are stabilized with intrachain bonds, including disulfide bonds, but the holoprotein is held together by noncovalent bonds (hydrogen bonds and hydrophobic interactions). These prolamins are soluble in alcohol-water; their molecular masses range from 30 to 60 kDa. In other prolamins (the former glutelins), monomers are held together not only by intrachain, but also by interchain disulfide bonds. As a result, they form some of the largest proteins known; the molecular mass of wheat or barley holoproteins may range from 11 to 133×10^2 kDa. These prolamins require reducing agents for solubilization.

After translation, the protomers of individual polypeptides in a prolamin are inserted into the ER, the signal peptide is cleaved, and disulfide linkages, both intra- and interchain, occur within the ER, but the further assembly and transfer to storage vacuoles in cereals follow two different routes (see Fig. A3-13). Prolamins and legumin-like proteins in some cereals (e.g., wheat, oat, and rice), after processing in the ER, are taken via the Golgi and *trans*-Golgi network to protein storage vacuoles, as in dicots. In contrast, in maize, nascent proteins are retained within the ER in special vesicles, which bud off from the ER; these vesicles coalesce by a process known as autophagy to form a new protein storage vacuole. Data indicate that at least some proteins in wheat and rice are also secreted into ER vesicles, which aggregate to form a PSV, as in maize.

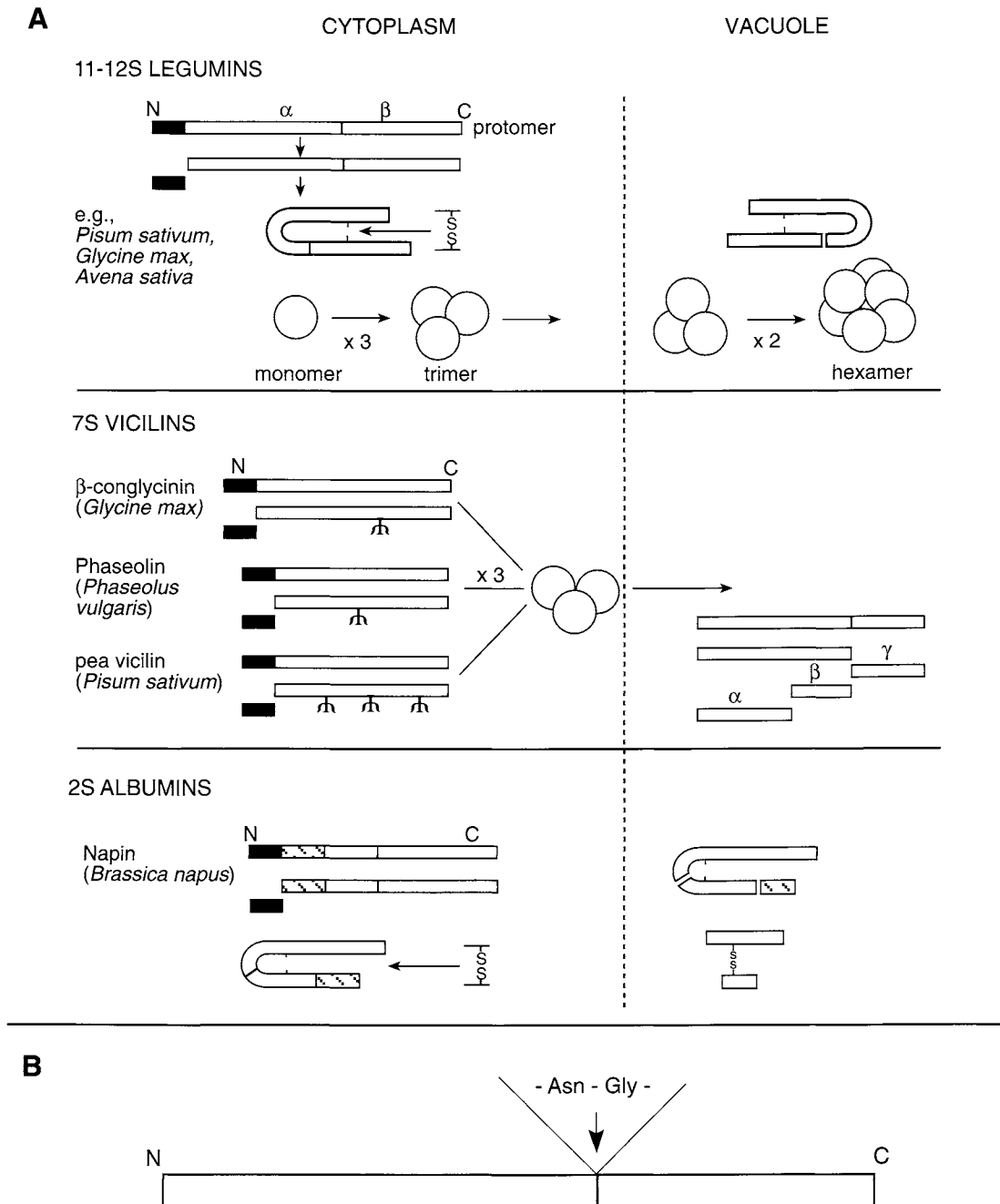


FIGURE A3-12 (A) Processing and assembly of 11-12S legumins, 7S vicilins, and 2S in dicots. Signal peptides are shown as solid black. Glycosylations are shown with an inverted Ψ . Propeptides are shown hatched. Adapted from Müntz (1996). (B) Cleavage of seed storage proteins in the vacuole occurs at a specific site, the peptide bond on the C-terminal side of an Asn residue of the preprotein precursor. The other amino acid is often, although not always, glycine. From data in Hara-Nishimura *et al.* (1995).

7.3. Enzymes

Many enzymes are involved in the processing and maturation of storage proteins before their storage and, subsequently, their hydrolysis following germination. These enzymes are not considered storage pro-

teins *sensu strictu*, but several of them are produced, transported, and stored in an inactive state, along with storage proteins, and activated *in situ* when needed. For example, cysteine endopeptidases are commonly involved in the digestion of storage proteins following seed germination. Some cysteine endopeptidases occur

in A and B forms (e.g., proteinase A and B). Whereas the B form may be produced during seed development and stored in the protein storage vacuoles, it is unable to attack the reserve proteins without preliminary limited proteolysis. This limited proteolysis is provided by form A, which is either newly synthesized at the time of seed germination or synthesized very late during embryogenesis and left as an inactive precursor and activated (or matured) at imbibition. Other cysteine endopeptidases are synthesized *de novo* after seed germination, but they may also have A and B forms that act cooperatively.

8. DIFFERENT FOOD RESERVES MAY BE ACCUMULATED AT DIFFERENT TIMES

As mentioned in Chapter 18, the deposition of reserve foods ("seed filling") generally starts with mid-embryogenesis, when the embryo and endosperm (in endospermic seeds) are in the expansion phase, and ends with the beginning of desiccation in late embryogenesis (see Fig. 18-1). Within this time frame, which varies for different species [e.g., for barley from about

5 to 15 days after pollination (DAP); for maize from 12 to 30 DAP; for *Arabidopsis* from 14 to 20 DAP], different foods may be elaborated and reach peaks of their deposition at different times. Moreover, some reserves may be deposited transiently. Thus, in many oilseeds, as also in wheat embryos, starch granules are deposited early in seed filling and then are hydrolyzed to provide carbon for the onset of the major deposition of oil bodies.

The deposition of different reserve proteins within a seed may also start and peak at different times with varying degrees of overlap. This can be shown by monitoring the expression patterns of transcripts for various seed storage protein genes. In many species, vicilins are accumulated ahead of legumins. Moreover, synthesis of the constituent subunits of a vicilin may start at different times. For example, the synthesis of α - and α' subunits of β -conglycinin in soybean begins about 15–17 days after flowering, but that of the β subunit does not start until 5–7 days later. This has important consequences for the degree of sulfation of the mature protein. Because α - and α' subunits have methionine, whereas the β chain does not, protein deposited early is more rich in methionine.

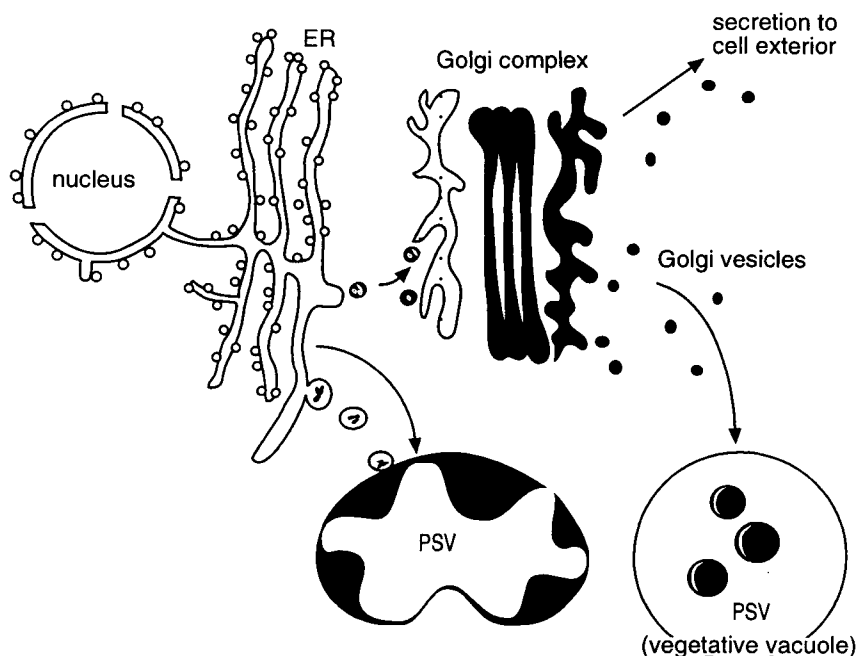


FIGURE A3-13 Two modes of transport of storage proteins to vacuoles in cereals. Proteins are synthesized on the ER and processed in the ER and Golgi complex. They are transported via Golgi vesicles to preexisting vacuoles (vegetative vacuoles), which become protein storage vacuoles (PSVs). In an alternative pathway, proteins are retained within the ER, packaged into ER vesicles, and released into the cytoplasm where they fuse to form a new vacuole, the PSV. Adapted from Okita and Rogers (1996).

Nutritional conditions, such as the availability of nitrogen and sulfur, during seed filling affect the types of reserves that may be synthesized. Thus, lack of nitrogen during seed filling may favor the deposition of starch or oils over that of proteins. Similarly, in plants growing in soil depleted of sulfur, seed proteins that are not sulfated, vicilins rather than legumins, are favored to accumulate.

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IV

MOLECULAR BASIS OF HORMONE ACTION

Plant responses to hormones involve many different types of actions—opening or closing of ion channels, cytoskeletal changes, differential gene expression, etc. All require that the hormone be recognized or perceived by the target cell or tissue. Because plant hormones are small, relatively simple molecules that lack the structural complexity and information content required for mediating diverse responses, the concept has grown that for each hormone there is a specific receptor (or receptors) that, on the one hand, recognizes the hormone and, on the other, initiates a sequence of events that result in the overt response.

Since small differences in the structure of the hormone, e.g., α vs β position of a specific atom or group, an extra $-\text{OH}$, or the presence of a $-\text{COOH}$ group or its methylation, can cause profound changes in biological activity of the hormone, it follows that the hormone–receptor interaction is highly stereospecific and that the hormone (or ligand following enzyme/substrate terminology) must have a precise three-dimensional fit with the receptor. Only those molecules that have the correct three-dimensional structure and the required complex of bonds and hydrophobic patches “fit” the receptor and are recognized. Analogs that are less biologically active fit less and are recognized less, and inactive analogs do not fit and are not recognized. Since, among the various macromolecules, only proteins have the required structural specificity, it is commonly assumed that the receptor, or at least the part of the receptor that is involved in recognition of the hormone, is a protein.

While the above concept of a receptor implies a precise three-dimensional fit between the hormone and its receptor, many data suggest that plant hormone receptors may be more catholic in accepting a wider variety of hormones or hormone

analogs. For instance, the molecular structures of biologically active auxins, indoleacetic acid (IAA), 1-naphthalene acetic acid (1-NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) are noticeably different one from the other, yet they all bring about auxin-type responses, albeit to varying degrees. There are several possible explanations: (i) Synthetic auxins act by increasing the endogenous IAA concentration either by promoting new synthesis or by inhibiting IAA conjugation or breakdown. This idea has been current for many years without any critical evidence to support it. (ii) There is more than one auxin receptor. (iii) The auxins are recognized more on the basis of charge distribution over the molecule rather than on the basis of a specific atom or group of atoms. Indeed, a charge separation theory was postulated in the 1960s to account for activities of structurally diverse auxins.

The situation with auxins is not unique. Structurally diverse natural or synthetic analogs with similar biological activities are known for abscisic acid (ABA), gibberellins, and brassinosteroids. So the alternatives are that there is more than one receptor for each of these hormones or, if there is a single receptor, that it recognizes not just one or more specific functional groups in the molecule, but the spatial distribution of all the functionalities present in it, i.e., the whole molecule. Because the receptors for plant hormones, except for ethylene, are still unknown, the issue cannot be resolved.

There are two prevailing concepts as to where the plant hormonal signal is perceived. One view states that the signal is perceived at the cell surface, presumably at the plasma membrane, and then is transmitted to the site of action *via* a series of steps, resulting ultimately in a physiological/biochemical response. The second view states that the site of signal perception is intracellular. There is greater evidence for cell surface perception for several hormones, although our present knowledge is not sufficient to exclude a perception in the cell interior. It is possible that both models apply. Some hormones may have a plasma membrane-based receptor, whereas others may have a cytoplasmic, soluble receptor. It is also possible that for a specific hormone, some events are regulated by a plasma membrane-based receptor, whereas the other events are regulated by a soluble receptor.

The perception of the hormonal signal and transmission of the signal to the site of action (signal transduction) are among the most intensively investigated areas of plant biology. For dissection of plant hormone signaling, three approaches have been used with varying degrees of success for each hormone. The most direct approach is to isolate the receptor protein using a labeled hormone or a close analog and purify it. This was the approach used for five major hormones from the early 1970s through the mid-1980s, but as explained in the following chapters, the approach has proven far more difficult than anticipated earlier and has not been particularly fruitful for most hormones.

Another approach is to isolate and clone genes that are specifically regulated at the transcriptional level by a hormone. Gene transcription is usually the culminating event in a signal transduction chain, and it is reasoned that if a sufficient number of such genes are available, the information from their common regulatory sequences and transcription factors (see Appendix 1) may allow working backward in the chain toward the receptor.

The third approach is to screen for mutants that show an altered sensitivity to a hormone in one or more specific responses. These mutants, known as response mutants, are unable to respond as expected because of a defective protein somewhere in the signal perception or transduction chain. Response mutants have become the method of choice for the dissection of signal perception/transduction pathways because (i) they can be specific to a particular signaling pathway and (ii) the isolation of mutant alleles allows cloning of the wild-type gene, and thus a clue as to the function of the encoded protein (see Appendix 1). However, no single approach is sufficient and, in order to prove that one is dealing with a receptor, data from all three approaches must be combined.

In Section IV, the molecular basis of ethylene action is covered first because it is the most completely known. Subsequent chapters deal with the molecular biology of action of auxins, ABA, and the remaining hormones. A fifth chapter on signal perception and the transduction paradigm, as known from yeast, animal systems, and plants, puts the previous chapters in perspective. An appendix on methodologies for hormone-binding assays and protein purification is provided at the end and concludes Section IV.

In reading these chapters it should be kept in mind that signaling pathways are not linear, but rather a network of branching and intersecting pathways where a straight cause and effect are rare, and one stimulus need not necessarily give the anticipated response. Although this may be intuited for living organisms, the demonstration that it is indeed so is a testimony to the patient efforts of scores of plant biologists (physiologists, biochemists, and molecular biologists) and a triumph of modern genetic and molecular techniques.

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Ethylene Signal Perception and Transduction

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1. ETHYLENE EFFECTS ON PLANT GROWTH AND DEVELOPMENT

Earlier chapters showed that ethylene plays multiple roles in plant growth and development: seed germination and seedling growth, rooting and root hair formation, epinastic growth, fruit ripening, and senescence and abscission of mature leaves, flowers, and fruits (Chapters 11, 14, 15, 17, and 20). Ethylene is also one of the hormones that enables plants to cope with stress. Stresses of various kinds, flooding, wounding, and pathogen invasion, induce ethylene synthesis, which in turn elicits adaptive changes in plant development. For example, the elongation growth of deep water rice is triggered in the first instance by ethylene. Likewise, the induction of cell death leading to the formation of air-conducting channels in water-logged terrestrial plants is traceable to ethylene. Ethylene is also responsible for induction of specific defense-related proteins, such as chitinases, proteinase inhibitors I and II, glucanases, and hydroxyproline-rich glycoproteins.

Most of the responses just given involve changes in gene expression, ion channel activities, cytoskeletal rearrangements, and alterations in patterns of cell growth and enlargement. This chapter deals with perception of the ethylene signal and its transmission to the point of gene expression. A model for ethylene signaling is presented.

2. ETHYLENE-BINDING STUDIES

Structure-activity relationships of ethylene and related compounds, as deduced from bioassays, show that small aliphatic compounds with two carbons, a double bond, and terminal carbon free of positive charge have much better activity than molecules with side groups, or triple bonds (Table 21-1).

Since silver ions inhibit ethylene action and since unsaturated aliphatic compounds, such as ethylene, bind to metals readily, it has been generally accepted since the 1960s that the receptor for ethylene has a metal-containing site, and recent work has established conclusively that the metal is copper.

In the 1970s and 1980s a good deal of work was done on [¹⁴C]ethylene binding using several plant tissues (for hormone-binding assays, specificity of binding, calculations of binding affinity and number of binding sites, and purification of binding proteins, see Appendix 4). These studies revealed the presence of high-affinity binding sites in membrane fractions that could be displaced or exchanged by biologically active analogs in proportion to their biological activity. However, the isolation of these sites, using biochemical techniques, proved elusive and, until recently, no direct connection could be established between ethylene binding and any physiological response.

3. ETHYLENE-INDUCED GENE EXPRESSION

Many putative ethylene-regulated genes have been cloned and sequenced. They are from three main

TABLE 21.1 Relative Activities of Ethylene and Related Compounds in Bioassays^a

Compound	Structure	Relative activity
Ethylene	$\text{CH}_2 = \text{CH}_2$	1
Propylene	$\text{CH}_3\text{CH} = \text{CH}_2$	100
Vinyl chloride	$\text{CH}_2 = \text{CHCl}$	1,400
Carbon monoxide	CO	2,700
Acetylene	$\text{CH} \equiv \text{CH}$	2,800
Vinyl fluoride	$\text{CH}_2 = \text{CHF}$	4,300
Propyne	$\text{CH}_3\text{C} \equiv \text{CH}$	8,000
Vinyl methyl ether	$\text{CH}_2 = \text{CH} - \text{O} - \text{CH}_3$	100,000
1-Butene	$\text{CH}_3\text{CH}_2\text{CH} = \text{CH}_2$	270,000
Carbon dioxide	CO_2	300,000

^aBased on data in Burg and Burg (1967).

systems: genes induced in pathogenesis-related (PR) responses and/or wound response, genes induced during ripening fruits (e.g., tomato, avocado), and those induced in senescing flowers (e.g., carnation petals).

Pathogenesis-related genes seem to be regulated differently than those associated with fruit ripening or senescence. For pathogenesis-related genes (e.g., chitinase, β -1,3-glucanase), an ethylene response element (ERE) consisting of a repeat GCCGCC motif has been identified. Some nuclear proteins that bind to the GCC motif are known from some plants, e.g., tobacco, and the steady-state levels of their mRNAs rise rapidly after ethylene treatment. Sequence analysis of these ERE-binding proteins (EREBPs) suggests that they belong to a large group of transcription factors found so far only in plants. Although individually variable, these proteins share a conserved domain of 59 amino acids, known as the AP2 domain, which is rich in charged amino acids and contains the DNA-binding site. Members of the AP2 family of transcription factors serve diverse functions in plants: some regulate flower development (e.g., APETALA2, AINTEGUMENTA2), some regulate the transcription of genes induced by environmental factors, such as cold temperature or drought, and some regulate pathogenesis-related genes.

Genes related to senescence or fruit development show no GCC motif in their promoters, but a precise definition of EREs has not yet been possible for these genes. For the tomato *E4* gene, which is ripening specific, it was shown that two *cis* elements, which act in concert, are necessary for ethylene responsiveness. The *E8* gene in tomato is induced independently by ethylene and by developmental signals, and different *cis* elements are required for induction in each case. The functions of these genes in tomato fruit ripening are unknown. Footprinting assays have shown that several nuclear proteins bind to the promoter sequences of *E4* and/or *E8* genes in tomato and to the promoter sequences of a glutathione *S*-transferase (*GST1*) gene in carnation petals; some transcription factors identified via ethylene response mutants have been shown to bind to promoters of *E4* and *GST1* genes (see Section 5.3).

ACC synthase and ACC oxidase genes, involved in ethylene biosynthesis (see Chapter 11), are induced by a variety of signals, developmental cues, stress, pathogen attack, and hormones such as auxins, cytokinins, or ethylene itself. Although some *cis* sequences have been described in promoters of these genes, it is still unclear as to which elements, singly or in combination, are responsive to which factor.

While the studies on ethylene binding have not been very fruitful and those on ethylene-induced gene expression have lagged behind those for some other hormones, the picture is dramatically different for the response mutants. These mutants have been most useful in elucidation of the ethylene signaling pathway, so much so that ethylene signaling now serves as a model system for research on signaling by other hormones. The elucidation of this pathway is due to the efforts of many scientists, especially Tony Bleecker at the University of Wisconsin, Madison; Joe Ecker at Salk Institute, San Diego (formerly at the University of Pennsylvania, Philadelphia), and Elliot Meyerowitz at California Institute of Technology, Pasadena, and their associates.

4. ETHYLENE RESPONSE MUTANTS

Seedlings of dicot plants grown in the dark and given ethylene (10–100 ppm) show short, swollen epicotyls/hypocotyls and a tight apical hook—the so-called “triple response” to ethylene. In contrast, seedlings grown in air are tall, slender, and show a much less tightened hook (see Chapter 11). Since the triple response in dark-grown dicot seedlings is a very characteristic ethylene response, it has been the basis for the selection of ethylene response mutants. Mutagenized seeds are plated for germination in the dark, either in the presence of saturating concentrations of ethylene or in the presence of air. Potential ethylene-insensitive mutants are tall with open hooks in the presence of ethylene. In contrast, potentially constitutive mutants show the triple response when grown in air (see Fig. 21-1). Both mutant phenotypes are easily picked out amid neighbors that show the wild-type response.

4.1. Ethylene-Insensitive Mutants

Several ethylene-insensitive mutants in *Arabidopsis* have been identified (see Table 21-2). These include *etr* (ethylene-resistant) and *ein* (ethylene-insensitive) mutations. These mutations were discovered by two different groups of researchers; hence, the different names. Some other mutants, known as *eti* (for ethylene insensitive) mutants, were discovered in a screen that correlated the ethylene sensitivity of mutagenized seedlings to their ability to push through compacted sand; those that were ethylene insensitive were less able to push through. These mutants are not well characterized and are not discussed any further.

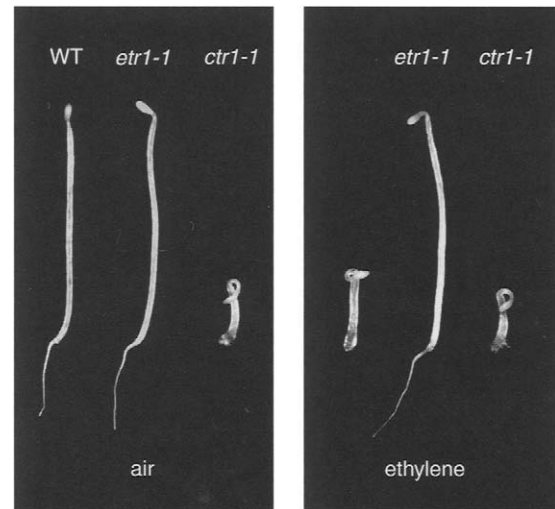


FIGURE 21-1 Ethylene response mutants of *Arabidopsis*. *Arabidopsis* seedlings are grown in the dark in the absence (left) or presence (right) of 10 ml per liter ethylene. The wild type (WT) develops the typical triple response phenotype in the presence of ethylene: a short and thick hypocotyl with an exaggerated apical hook and a short and thick root. The constitutive ethylene response mutant *ctr1-1* develops the triple response phenotype even in the absence of ethylene, whereas the ethylene-insensitive mutant *etr1-1* shows no triple response even in the presence of ethylene. From Chang and Stadler (2001).

Among the various loci for ethylene insensitivity in *Arabidopsis*, the *ETR1* locus was the first to be characterized, and more is known about it than any other similar locus. Hence, we will consider *ETR1* first and see how it is involved in ethylene signaling.

4.1.1. *ETR1* Locus

Four mutant alleles at the *ETR1* locus are known, *etr1-1* through *etr1-4*; all are inherited as dominant

TABLE 21-2 Ethylene Response Mutants in *Arabidopsis*^a

Mutants with pleiotropic effects					
Insensitive mutants		Constitutive mutants		Organ-Specific mutations	
<i>etr1</i>	d ^a	<i>eto1</i>	r	<i>hls1</i>	r
<i>etr2</i>	d	<i>eto2</i>	d	<i>eir1</i>	r
<i>ein4</i>	d	<i>eto3</i>	d		
<i>ein2</i>	sd				
<i>ein3</i>	sd	<i>ctr1</i>	r		
<i>ein5</i> (<i>ain1</i> for ACC insensitive)	r				
<i>ein6</i>	r				

^aInherited as a dominant (d), semidominant (sd), or recessive (r) trait. Adapted with permission from Ecker (1995), © American Association for the Advancement of Science.

traits. *etr1* mutants are strongly ethylene insensitive (Fig 21-2) and pleiotropic in their effects. In addition to insensitivity in the triple response of seedlings, the mutants show larger rosettes than the wild type, delayed senescence when leaves are detached or exposed to ethylene, delayed abscission, and epinasty. Ethylene-induced gene transcription is either absent or delayed. The endogenous levels of ethylene tend to be higher than in the wild type because the negative feedback control on ethylene synthesis is blocked in the mutant.

4.1.2. Other ETR and EIN Loci

etr2 and *ein4* are dominant mutations that are similar in phenotype and ethylene insensitivity to *etr1*. They could be mutations in other members of the same, or similar, gene families as *ETR1* (see Section 7).

In contrast to *etr1*, *etr2*, and *ein4*, several other insensitive mutations, *ein2* and *ein3*, are semidominant, whereas *ein5* (also known as *ain1* for ACC insensitive) and *ein6* are recessive mutations (see Table 21-2). Among these, *ein2* is a strong mutation. Homozygous *ein2* plants show many of the same insensitivities to ethylene as *etr1* plants and, like them, are larger and

have a higher endogenous content of ethylene than the wild-type plants (see Fig. 21-3). The remaining *ein* mutations are weaker than *ein2*, but show insensitivities to all ethylene responses.

4.2. Constitutive Mutants

Mutants that show a constitutive triple response fall into two categories.

4.2.1. *eto* Mutants

eto (for ethylene overproducing) mutants (see Table 21-2) show a constitutive triple response as seedlings because their endogenous content of ethylene is very high (Table 21-3). The effects of these mutations appear to be restricted to seedlings because ethylene levels in various organs of the adult plants are similar to those in the wild type. *eto* Mutants are restored to wild type if grown in the presence of ethylene synthesis inhibitors, such as aminoethoxyvinylglycine (AVG); hence, they are not mutations in the response pathway and are not considered any further in this chapter.

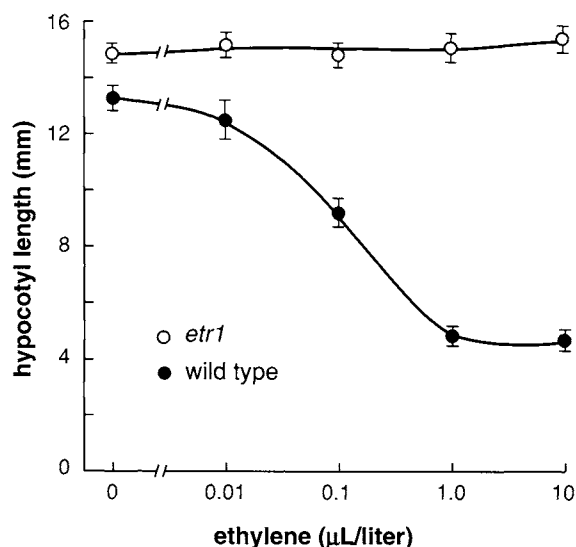


FIGURE 21-2 The effect of increasing concentrations of ethylene on the hypocotyl length of wild-type (WT) and *etr1* seedlings grown in darkness. Note that *etr1* seedlings are unaffected by ethylene concentrations as high as $10.0 \mu\text{L} \cdot \text{liter}^{-1}$ (even $100 \mu\text{L} \cdot \text{liter}^{-1}$, not shown), whereas WT seedlings show a 70% reduction in hypocotyl length at $1.0 \mu\text{L} \cdot \text{liter}^{-1}$. For this experiment, seeds were germinated on 1% agar in sealed 1-liter containers into which ethylene was injected to give the concentration indicated. Ethylene was renewed every 12 h. Hypocotyl length was measured after 3 days of growth. Each point represents the mean of 10 measurements \pm SE. Reprinted with permission from Bleecker *et al.* (1988), © American Association for the Advancement of Science.



FIGURE 21-3 Phenotypes of wild-type, *etr1*, *ctr1*, and *ein2* *Arabidopsis* seedlings in air and ethylene. From Ecker (1995).

TABLE 21-3 Ethylene Production in Wild-Type and Constitutive Mutants of *Arabidopsis*^a

Strain		Seedlings ^b
Wild type		0.4 ± 0.1
<i>eto1-1</i>	r	3.5 ± 0.6
<i>eto2</i>	d	7.7 ± 1.5
<i>eto3</i>	d	35.0 ± 3.5
<i>ctr1-1</i>	r	0.1 ± 0.1

^aMutations are recessive (r) or dominant (d). Ethylene levels are expressed as nanoliters of ethylene per 500 seedlings. Modified with permission from Kieber *et al.* (1993), © Cell Press.

4.2.2. *CTR1* Locus

The second type of mutants, *ctr1* (for constitutive triple response 1), show the triple response when grown in air, but, nonetheless, have a normal endogenous content of ethylene (see Table 21-3). Use of ethylene biosynthesis inhibitors does not restore them to wild type.

ctr Mutations have been defined so far at one locus only; hence, they are referred to as *ctr1*. Five mutant alleles have been isolated, and genetic analysis shows that all are recessive. *ctr1* Mutant seedlings and adult plants show a phenotype that resembles wild-type plants that are constantly exposed to saturating concentrations of ethylene (see Fig. 21-3). They are pleiotropic in all the measured ethylene responses. The apical hook remains closed longer when plants are transferred from darkness to light; all organs (leaves, inflorescence, roots) are much more compact due to a smaller cell size than the wild type; the plants require a longer time for bolting. The production of root hairs in epidermal cells of *Arabidopsis* roots is highly patterned. This pattern is disturbed in *ctr1* mutants, and epidermal cells that are not programmed to produce root hairs produce them ectopically. As a result, the roots, although shorter, produce far many more roots hairs than in the wild type. Moreover, in *ctr1* plants, the steady-state levels of RNAs for some ethylene-induced genes (e.g., *EI305* gene and a chitinase gene) are constitutively high, as in wild-type plants grown in the presence of ethylene.

4.3. Organ-Specific Mutants

In addition to the mutants just described, which are pleiotropic in their effects, some mutants show ethylene-insensitivity in specific organs. Two such mutations, *hls1* (for hookless 1) and *eir1* (for ethylene insensitive root) represent lesions in auxin homeostasis and/or transport, but were initially isolated in screens for ethylene insensitivity. Mutant seedlings

carrying strong alleles of *hls1* do not form an apical hook when grown in darkness and exposed to ethylene, though they remain sensitive to ethylene inhibition of root or hypocotyl elongation. The *eir1* mutants are insensitive to ethylene in root elongation and gravitropism, but the hypocotyl and hook are ethylene-sensitive. *EIR1* as mentioned elsewhere encodes an efflux carrier for basipetal transport of auxin in roots (see Chapter 13). The *hls1* mutant has the phenotype obtained when wild-type seedlings are treated with auxin transport inhibitors; it is discussed in Chapter 15, Section II, 7.1.

4.4. Response Mutants Indicate the Ethylene Signaling Pathway

As explained in Appendix 1, genetic analysis is used to determine the order in which two gene products act in a particular pathway. An analysis of double mutants *etr1-1 ctr1* or *ein4 ctr1* indicates that CTR1 acts downstream of both ETR1 and EIN4. In double mutants of *ctr1* with *ein2*, or any other weaker *ein* mutant, an *ein* phenotype is seen, which indicates that all these EIN products (except EIN4) act downstream of CTR1.

Genetic analysis is also used to determine whether two gene products act in the same pathway or in separate pathways that converge (see Appendix 3-1). Double mutants between *etr1* and *ein2* do not display an additive phenotype, which indicates that the two mutations function in the same pathway. Also, double mutants between *ein2* and the weaker *ein* mutants display the *ein2* phenotype, indicating that they all function in the same pathway.

Based on these genetic studies, it has been proposed that ethylene signaling occurs *via* a linear pathway, in which ETR1 and/or EIN4 act upstream of CTR1. EIN2 acts downstream of CTR1, and the other EIN products (e.g., EIN3, EIN5, EIN6) act further downstream of EIN2 (Fig. 21-4). The HLS1 and EIR1 gene products are organ specific and represent branch points in ethylene signaling. They may also interact with elements in auxin (and other) signaling pathways.

5. CLONING OF WILD-TYPE GENES AND STRUCTURES OF ENCODED PROTEINS

The cloning of *CTR1* and *ETR1* genes, both in 1993, was a milestone in plant hormone research: (1) it provided for the first time a probable candidate for a plant hormone receptor and (2) it indicated a possible

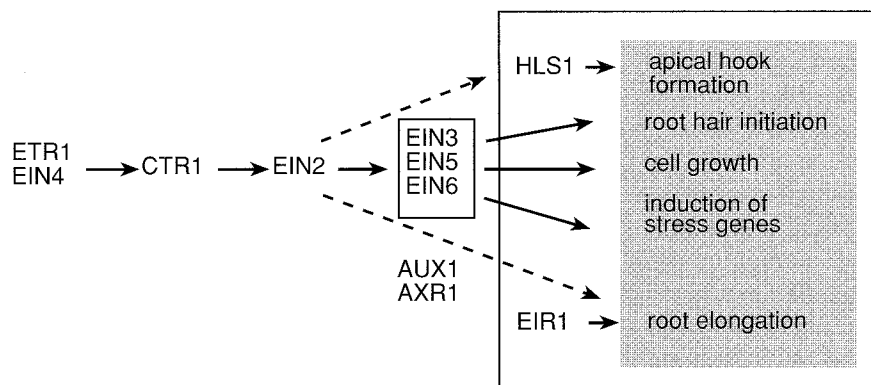


FIGURE 21-4 Ethylene signaling pathway deduced from genetic analysis of response mutants in *Arabidopsis*. Although the order in which these gene products act is clear, there may be one or more intermediates between any two of these products. Broken arrows indicate interaction with other signaling pathways. Adapted from Kieber (1997).

pathway by which hormone signaling in plants may be affected.

5.1. Cloning of *ETR1* Gene

The *ETR1* gene was cloned by positional mapping and chromosome walking. Sequence analysis of the

encoded protein, ETR1, shows that it combines features of the prokaryotic two-component signaling systems (see Box 21-1). As shown by Northern hybridizations, *ETR1* mRNA is expressed in all parts of the plant and such expression is independent of ethylene treatment.

BOX 21-1 TWO-COMPONENT SIGNALING SYSTEMS

PROKARYOTES RESPOND TO CHANGES in their environment, such as N₂ or P availability, chemosensory stimuli, osmotic stress, and oxygen tension, by a communication system, which has been well analyzed. A superfamily of proteins participates in this communication system, often referred to as the two-component system. There may be as many as 50 different types of two-component systems within a prokaryotic cell.

Each two-component system has two separate proteins: the sensor and the associated response regulator (Fig. 21-5A). The sensor protein has an extracellular domain that acts as the receptor for the signal, this is the signal input domain. It is connected to an intracellular, cytoplasmic domain, which is highly conserved and acts as a protein kinase. The kinase is almost invariably a histidine protein kinase. This domain is called the transmitter. The response regulator, like the sensor, has two domains: a receiver module that takes the signal from the histidine kinase and an output domain, which, in many cases, acts as an activator of transcription factors.

On receiving the signal, the sensor undergoes a conformational change and the transmitter domain is autophosphorylated, using ATP as the phosphate donor, at the conserved His residue (Fig. 21-5B). The phosphorylated His, in turn, transfers the phosphate group to a conserved Asp residue in the receiver module. The response regulator may then interact with transcription factors directly or *via* a series of secondary signaling molecules.

This arrangement offers a highly efficient mechanism by which prokaryotes sense and respond to changes in their immediate environment. Both the signal input domain of the sensor protein and the activator domain of the response regulator are, as expected, highly variable. They are designed to sense different stimuli on the one hand and to elicit expression of different genes on the other.

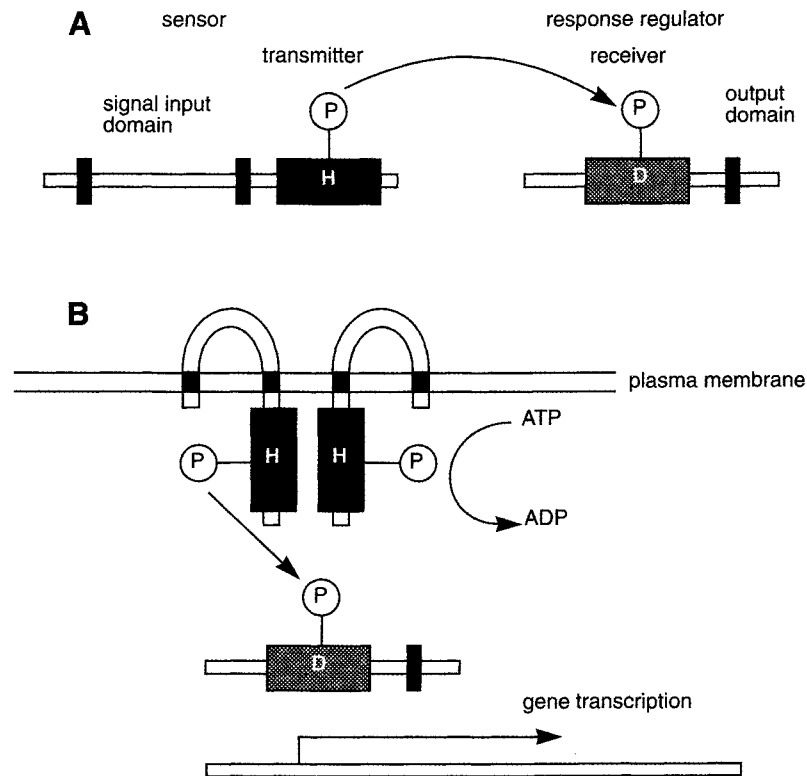


FIGURE 21-5 Prokaryotic two-component signal transduction system. A “simple” system involving a single phospho transfer is shown. (A) The sensor protein is shown with two membrane-spanning regions (vertical bars) and the conserved histidine kinase (H) domain. The response regulator has a receiver domain with a conserved aspartate residue (D) and an output domain, which may interact with transcription factors via the DNA-binding domain (vertical bar). Transfer of the phosphate group (P) from His to Asp is shown. (B) Action of the two-component system. The sensor protein is anchored in the plasma membrane and is shown as a dimer. The extracellular domain senses the environmental signal, which results in autophosphorylation of the histidine kinase, using ATP as the phosphate donor. The phosphate is then transferred to the aspartate in the receiver, which activates the output domain. Although both His residues are shown as phosphorylated, only one is shown transferring the phosphate group to Asp in the receiver module. The final phospho acceptors in prokaryotic two-component systems are usually, but not always, transcriptional regulators. Adapted from Wurgler-Murphy and Saito (1997).

A subset of the just-described two-component system is seen in some prokaryotes. In this subset, the sensor, including the histidine kinase, and the receiver part of the response regulator occur on the same polypeptide, with His and Asp on the same C-terminal, but separated by a stretch of amino acids.

The two-component systems and variants thereof, although discovered in bacteria, are now known from many eukaryotes, including slime molds, fungi, and plants. Yeast (*Saccharomyces cerevisiae*) cells, growing on fruits, are subjected to rapid changes in osmolarity as starch is hydrolyzed to sugar or as the sugar concentration increases during fruit desiccation. Yeast cells use a modified version of the two-component system to detect changes in osmolarity of the external medium and turn on the synthesis of a compatible osmolyte, in this case, glycerol. This response, known as the high osmolarity glycerol (HOG) response, involves the activation of transcription factors for the gene encoding the key enzyme in glycerol biosynthesis, the NAD⁺-dependent glycerol-3-phosphate dehydrogenase (GPD).

Changes in osmolarity are sensed by a transmembrane protein, SLN1, which is a modified version of a two-component system. The SLN1 protein combines the histidine kinase domain of the sensor and the aspartate residue of the response regulator in the same polypeptide (Fig. 21-6A), but the SLN1 protein does not transfer the phosphate group directly to transcription factors. Instead, this transfer involves many proteins. Autophosphorylation of the histidine residue is followed by transfer of the phosphate group

from the Asp of SLN1 through the His residue of an intermediate to Asp of another protein, SSK1. SSK1, in turn, transfers the phosphate group to a phospho transfer cascade that is unique to eukaryotes, known as the mitogen-activated protein kinase (MAPK) cascade (Fig. 21-6B).

Mitogen-activated protein kinases in eukaryotes belong to another class of protein kinases, the serine/threonine protein kinases. The Ser/Thr protein kinases are a large superfamily of protein kinases with many subfamilies. Members of one subfamily, the Raf (or MAPK) family, occur ubiquitously in mammals, chicken, and fruit fly and have been shown to mediate various cellular activities, including cell division, cell growth, and differentiation, by transducing signals from cell surface receptors to transcription factors (Fig. 21-7). They are now known from yeast and plants as well. The MAPK cascade begins by the transfer of a phosphate group from a donor, such as SSK1 in yeast, to MAP kinase kinase kinase (MAPKKK), which transfers the phosphate group to MAP kinase kinase (MAPKK), which transfers the phosphate to MAP kinase (MAPK). MAPK, the last protein kinase in the chain, may activate transcription factors by phosphorylation or donate the phosphate group to some other protein in the signaling chain.

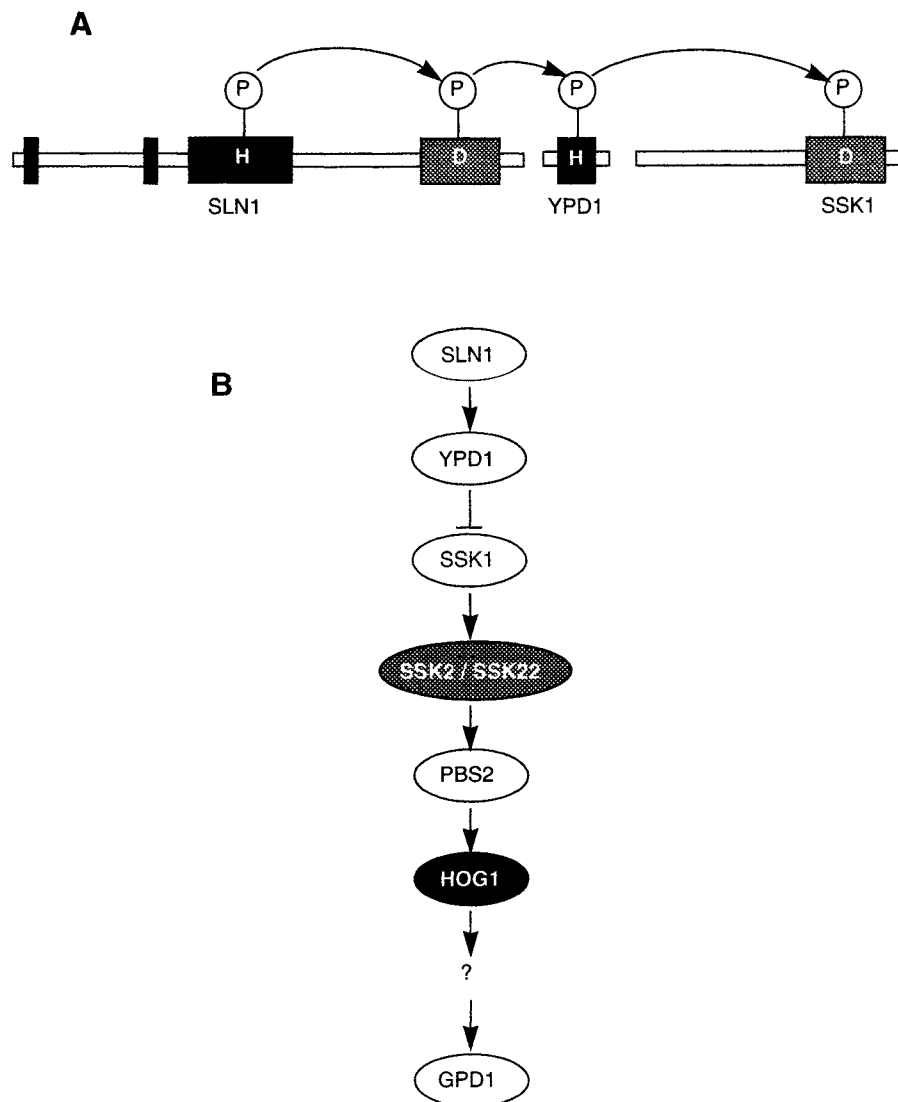


FIGURE 21-6 Osmoregulation in yeast (*Saccharomyces cerevisiae*). (A) The SLN1 protein combines the histidine kinase domain of the sensor protein and the conserved aspartate residue of the response regulator on the same polypeptide. The signal is then transmitted *via* a multistep phospho relay involving YPD1 and SSK1, ultimately to a MAPK cascade. (B) Elements of the MAPK cascade in yeast. Adapted with permission, from Wurgler-Murphy and Saito (1997), © Annual Reviews.

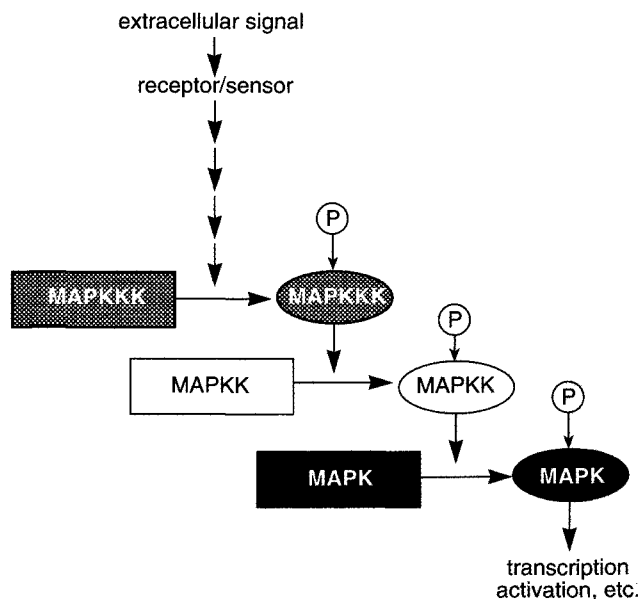


FIGURE 21-7 Signal transduction by the mitogen-activated protein kinase (MAPK) cascade. Extracellular stimuli are recognized by specific receptors/sensors on the cell surface and generate intracellular signals, which in one type of signaling are transmitted to MAPK cascades. A number of distinct MAPK cascades may coexist in a cell. Activated MAPKKK phosphorylates and activates a specific MAPKK, which in turn phosphorylates and activates a cognate MAPK. Activated MAPK may migrate to the nucleus and phosphorylate specific transcription factors and thus induce gene expression. Adapted with permission from Wurgler-Murphy and Saito (1997), © Annual Reviews.

The MAPK cascade is only one mechanism by which extracellular signals perceived by the receptor/sensor at the cell surface are transduced. There are other mechanisms as well, which are considered in Chapter 25.

An individual eukaryotic cell has many different species of MAPK cascades that participate in specific signaling events. Each is probably activated by a distinct phospho donor, although the exact mechanism of activation is not understood. The MAPK cascade specific for the HOG response in yeast starts with phospho transfer from SSK1 to SSK2/SSK22, a yeast MAPKKK, and ends with HOG1, an MAPK, which, on activation, is translocated to the nucleus, where it phosphorylates one or more transcription factors that induce *GPD1* and other genes (see Fig. 21-6B). Protein phosphatases are also involved in signaling and can provide negative or positive regulation by dephosphorylation (see Chapter 25). Thus, HOG1 can be dephosphorylated by a specific phosphatase to provide negative regulation.

ETR1 protein is structurally similar to SLN1 or a modified version of the prokaryotic two-component signaling system, whereas CTR1 is similar to a MAPKKK (see Section 5.2). Thus, ethylene signaling involves an unusual, although not unique, combination of features from prokaryotic as well as eukaryotic signaling systems.

The ETR1 polypeptide has an N-terminal region, which includes three regions rich in hydrophobic amino acids, all transmembrane domains, and an intracellular C-terminal region, which combines the functions of the kinase domain of the sensor protein and the receiver domain of the response regulator, as seen in the SLN1 protein in yeast and in modified versions of the two-component signaling system in bacteria (Fig. 21-8).

All four mutant alleles, *etr1-1* to *etr1-4*, have single amino acid changes within the transmembrane segments. Although all four mutant alleles are dominant over the wild-type allele, dose-response curves indicate that *etr1-1* and *etr1-4* are stronger alleles; they completely abolish all ethylene responses, whereas *etr1-2* and *etr1-3* show a reduced responsiveness to ethylene.

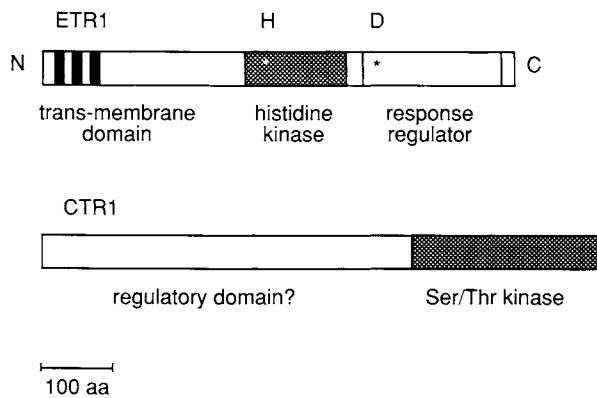


FIGURE 21-8 Schematic representations of ETR1 and CTR1 proteins. The ETR1 has an extracellular region and three transmembrane domains at the N-terminal, which connect to an intracellular region that carries a histidine kinase domain and, further toward the C terminus, a response regulator with a conserved aspartate residue. The conserved His and Asp residues are indicated by an asterisk. The CTR1 protein carries a highly conserved carboxy terminus, which acts as a serine/threonine protein kinase. The bar in the lower left shows a scale in amino acids. Adapted from Kieber (1997).

5.2. Cloning of the *CTR1* Gene

The *CTR1* gene was isolated and cloned using a T-DNA-tagged allele. Like *ETR1*, *CTR1* mRNA is expressed in all parts of the plant, and its expression is independent of ethylene treatment.

The amino acid sequence of the CTR1 protein is most similar to the Raf family of serine/threonine protein kinases. Raf-1 protein, an MAPKKK, has three domains: the most N-terminal domain has a binding site for Ras, a small GTP-binding protein, and a zinc finger domain; the middle domain is rich in serine/threonine residues, which may be a site for autophosphorylation or phosphorylation by other kinases; and the C-terminal domain is the kinase catalytic domain. It is believed that the N-terminal domain normally keeps the kinase activity of Raf-1 under check, an inhibition that is released when the amino terminal is phosphorylated by Ras. The carboxy terminal of CTR1 shows high homology to the kinase catalytic domain of Raf-1; it also has the serine/threo-

nine-rich tract, but lacks the Ras-binding site or the zinc finger.

All mutations in the *CTR1* gene that have been analyzed are in the catalytic domain and are predicted to disrupt its kinase activity. Because *ctr1* mutations are recessive, this suggests that the kinase activity of CTR1 is required to negatively regulate the ethylene response pathway. It also indicates that the kinase activity of CTR1 is active in air and is shut off in the presence of ethylene.

The immediate proteins on either side of CTR1, one that activates it by phosphorylation and one that is the target of the kinase activity, are unknown. As mentioned earlier, CTR1 lacks the Ras-binding site. It is possible that ETR1 interacts directly with CTR1. The amino terminal of CTR1 has been shown to bind the histidine kinase domain or the receiver domain of ETR1 in the yeast two hybrid assay and *in vitro* coimmunoprecipitation experiments. Also, several *Arabidopsis* cDNA clones with sequence similarities to a response regulator in a prokaryotic system have been isolated, and shown to participate in phospho-transfer from a histidine kinase in *E. coli*. These proteins may provide the link between ETR1 and CTR1. For downstream signaling, despite the fact that many MAPKKs and MAPKs are known from *Arabidopsis*, a specific connection with CTR1 has not been established, and there is still no direct evidence that CTR1 functions as a MAPKKK or that a MAPK cascade forms a component of ethylene signaling.

5.3. Downstream Signaling Elements

5.3.1. *EIN2* Protein

The *EIN2* gene is an important component of ethylene signaling, as is suggested by the fact that nearly all mutant alleles at this locus confer complete ethylene insensitivity. *EIN2* encodes a large protein (141 kDa, 1294 amino acids) with the N-terminal one-third consisting of 12 transmembrane domains, whereas the C-terminal two-thirds is cytoplasmic (Fig. 21-9). Transgenic expression of the transmem-

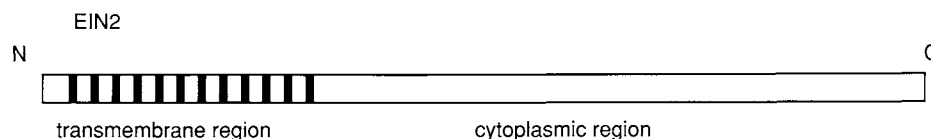


FIGURE 21-9 Structure of the *EIN2* protein. The N-terminal transmembrane region is predicted to have 12 α helices, which traverse the membrane, and a C-terminal cytoplasmic region. The extreme N terminus is also cytoplasmic. An amphipathic coiled-coil occurs in the cytoplasmic region near the last transmembrane domain. Adapted from Alonso *et al.* (1999).

brane and/or cytoplasmic regions indicates that EIN2 is a bifunctional protein. While the entire protein is essential for obtaining the triple response in dark-grown seedlings, various other ethylene responses, including the expression of *Arabidopsis* *GST1* and *BASIC CHITINASE* genes, can be obtained by expressing only the C-terminal two-thirds of the EIN2 protein in transgenic *ein2* mutants. Thus, the C-terminal two-thirds of EIN2 is sufficient to give a constitutive ethylene response. It is also sufficient to interact with jasmonate signaling. For example, wild-type *Arabidopsis* plants infected with pathogens produce both jasmonic acid and ethylene, and the production of both hormones is essential for induction of the defensin gene *PDF1.2*. The gene can also be induced by the simultaneous treatment of wild-type plants with the two hormones in the absence of the pathogen, but *ein2* plants fail to respond if so treated with methyl jasmonate. However, *ein2* plants transformed with the C-terminal two-thirds of EIN2 are able to respond normally and express *PDF1.2*. In addition, *ein2* mutant alleles have been picked up in screens for *Arabidopsis* mutants resistant to auxin transport inhibitors, cytokinins, or ABA and in screens for delayed senescence. Thus, EIN2 appears to be the site for cross-talk between ethylene signaling and signaling by other hormones (for ABA, see Chapter 23). Significantly, the cytoplasmic region of EIN2 has an amphipathic coiled-coil, which may be involved in protein-protein interactions.

5.3.2. EIN3 and Other Transcription Factors

Among the elements further downstream, the *EIN3* gene encodes a protein that has all the hallmarks of a transcription factor: nuclear localization signals (NLS), an acidic N-terminal domain, a proline-rich region, and a coiled structure involved in protein-protein interactions. Transient expression assays of *EIN3::GUS* construct in protoplasts show that EIN3 is localized in the nucleus. Finally, overexpression of the *EIN3* protein causes transgenic plants to show a constitutive ethylene response phenotype.

That EIN3 indeed serves as a transcription factor for ethylene responsive genes in *Arabidopsis* was demonstrated in an elegant series of experiments.

i. Sequence data from an ethylene response element-binding protein 1 (EREBP1) from tomato were used to isolate a similar protein from *Arabidopsis*. This protein, called ethylene response factor 1 (ERF1), was confirmed to bind to promoter sequences containing GCC boxes (e.g., of *BASIC CHITINASE* genes, *bCHI* and *CH5B*, but to no other).

ii. To confirm that ERF1 was involved in ethylene signaling, the coding sequence of *ERF1*, under the control of a constitutive promoter, was used to transform *Arabidopsis* plants in wild type as well as in *ein2* and *ein3* mutant backgrounds. Irrespective of the background, transformed plants showed an ethylene response phenotype in air, as well as in the presence of ethylene, showing clearly that ERF1 acts downstream of EIN2 and EIN3 and, by itself, is sufficient to induce ethylene-mediated responses (Fig. 21-10).

iii. Gel mobility shift assays using DNA fragments from the *ERF1* promoter and recombinant EIN3 protein expressed in bacteria showed that EIN3 binds specifically to a 36-bp fragment in the *ERF1*

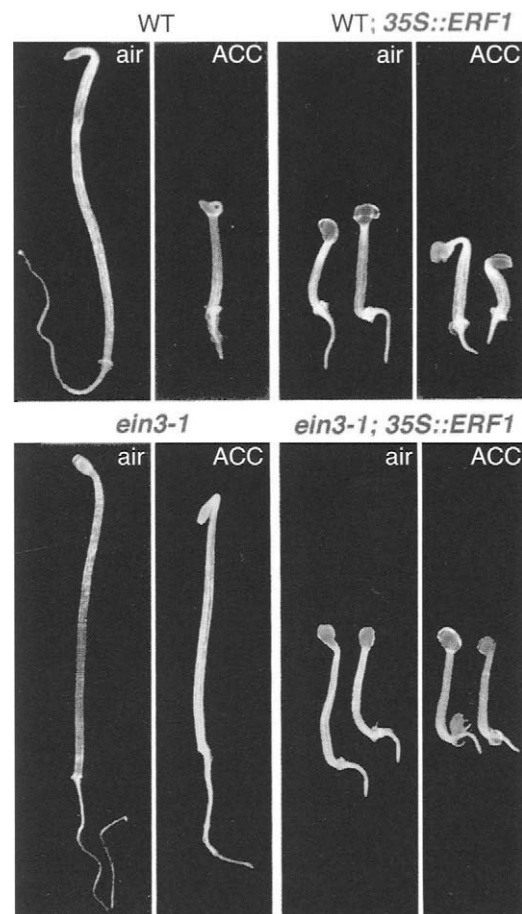


FIGURE 21-10 *Arabidopsis* seedlings transformed with the *EIN3* or *ERF1* gene in *ein3* background. *ein3* mutants show tall slender phenotype as expected. *ein3* mutant seedlings transformed with an *ERF1* coding sequence under the control of the 35S CaMV promoter show a triple response irrespective of whether they are grown in air or in ethylene. For ethylene treatment, seedlings were grown in the presence of ACC, which is taken up by roots and converted to ethylene. From Solano *et al.* (1998).

promoter and similar sequences in promoters of other ethylene induced genes, such as, tomato *E4*, and *ACC OXIDASE1* (*ACO1*) genes, and carnation *GST1* gene.

iv. Finally, full-length and truncated EIN3 products, obtained by *in vitro* translation, were used in gel mobility shift assays, and EIN3 was shown to bind to the *ERF1* fragment as a homodimer.

These results serve to link ethylene perception by an ETR1 type protein at the cell surface to ethylene-induced gene transcription via EIN3 and ERF1 (Fig. 21-11). They confirm that EIN3 is a transcription factor that regulates the expression of ERF1. ERF1 is also a transcription factor, which, in turn, regulates the expression of some pathogenesis-related genes, such as *BASIC CHITINASE* genes. EIN3-binding sites also occur in promoters of some other ethylene-induced genes, e.g., tomato *E4* and *ACO1* genes, and carnation *GST1* gene. The role of *E4* gene is not clear, but *ACO1* and *GST1* genes encode enzymes involved in ethylene biosynthesis and stress tolerance (in this instance, detoxification of xenobiotics), respectively. To date, *ERF1* gene is the only known direct target for EIN3.

It should be noted, however, that while *ERF1* is induced by ethylene, the *EIN3* gene is not. The possibility that it may be regulated by ethylene at a post transcriptional level remains open, however.

Several *EIN3*-like genes are present in the *Arabidopsis* genome. Three of these genes, *EIL1*–*EIL3* (*EIN3* like) have been cloned. They show sequence similarity to *EIN3* and have a similar domain structure. They may represent part of the repertoire of transcriptional regulators needed for tissue- and or response-specific expression of ethylene-induced genes (see Fig. 21-11). *EIN3/EIL* orthologs are beginning to be isolated from other plants as well (e.g., tobacco).

6. EVIDENCE THAT ETR1 IS AN ETHYLENE RECEPTOR

Since epistasis data show that ETR1 is the first protein in the ethylene signal perception–transduction chain, and mutations in the ETR1 protein abolish all sensitivity to ethylene in a global fashion, there is sufficient *a priori* reason to believe that ETR1 is an ethylene receptor. The structural similarity of ETR1 to SLN1 protein in yeast and to modified two-component systems in prokaryotes, both of which are involved in sensory perception and transduction of signal to intracellular elements, is strong supportive evidence. Some other data provide additional evidence that ETR1 is an ethylene receptor.

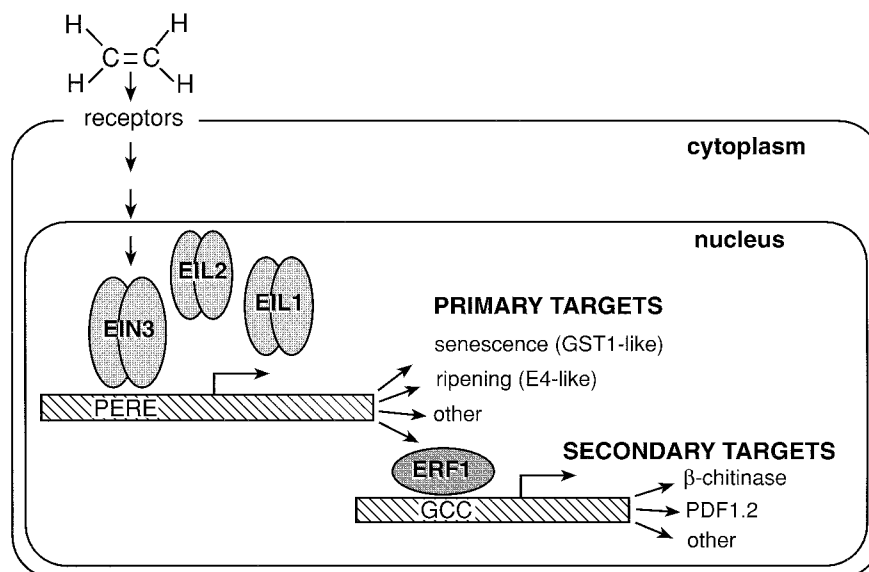


FIGURE 21-11 Model linking ethylene perception at the cell surface to regulation of gene transcription by EIN3/EIL and ERF1 proteins. The model shows EIN3 or EIL proteins directing the expression of ERF1 and other primary target genes (e.g., the *E4* gene in tomato) by binding as dimers to primary ethylene response elements (PERE) present in their promoters. ERF1 and probably other EREBPs bind to the GCC box in promoters of secondary target genes, such as *BASIC CHITINASE* and *DEFENSIN* (*PDF1.2*). Other more complicated models with intermediate proteins between EIN3 and ERF1 are possible. Reprinted with permission from Solano *et al.* (1998), © Cold Spring Harbor Laboratory Press.

6.1. [^{14}C]Ethylene Binding by ETR1

Ethylene-binding experiments indicate that *etr1* mutant plants specifically bind only about 20% of [^{14}C] ethylene as do wild-type plants (Table 21-4).

The *ETR1* gene and the mutated *etr1-1* allele were expressed in yeast cells. Yeast cells, by themselves or when transformed with the *etr1-1* mutant gene, show no [^{14}C] ethylene binding. However, when transformed with the wild-type *ETR1* gene, they show saturable binding (Fig. 21-12) with a calculated K_D of $0.04 \mu\text{l} \cdot \text{liter}^{-1}$ ethylene gas. This value agrees with the amount of ethylene required for the half-maximal response in the triple response bioassay. Furthermore, binding is inhibited by *trans*-cyclooctene and 2,5-norbornadiene, both competitive inhibitors of ethylene action in plants.

6.2. ETR1 Protein Functions as a Dimer

The ETR1 protein expressed in yeast or in transgenic *Arabidopsis* fractionates with membrane fractions. Monoclonal antibodies prepared against the protein indicate a molecular mass of 79 kDa on denaturing gels and 147 kDa on nondenaturing gels, which suggests that the protein occurs as a dimer in the natural state. Dimerization involves disulfide links and cysteine residues near the N-terminus (Cys⁴ or Cys⁶) have been implicated in dimerization (Fig. 21-13). However, the cytoplasmic receiver domain of ETR1 has been crystallized and found to occur as a dimer in crystal as well as in solution. Moreover, the carboxy terminus is reportedly involved in dimerization. The significance of these data, and the relationship between ethylene-binding, phosphorylation and dimerization of ETR1 are still unclear. Dimerization of

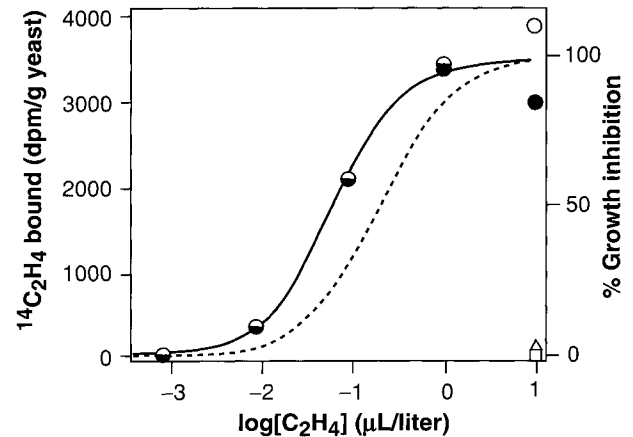


FIGURE 21-12 Specific binding of [^{14}C]ethylene by yeast cells transformed with full-length *ETR1* cDNA or its mutated allele. Duplicate samples of yeast expressing ETR1 (open and closed circles) were incubated with [^{14}C]ethylene at concentrations indicated on the abscissa plus/minus a thousandfold excess of unlabeled ethylene for 6 h and [^{14}C]ethylene bound saturably was plotted. For comparison, a dose-response curve for hypocotyl growth inhibition in *Arabidopsis* seedlings by ethylene is shown as a dotted line. Yeast cells transformed with the vector alone (\square) or the vector plus the mutant *etr1-1* allele (\triangle) show no binding. Reprinted with permission from Schaller and Bleeker (1995), © American Association for the Advancement of Science.

receptor proteins is thought to be important for signal amplification (see Chapter 25).

6.3. Anchoring of ETR1 in the Membrane Is Important for Ethylene Binding

It was mentioned earlier that all four *etr1* mutant alleles are point mutations in the putative transmembrane regions in the N terminus of the ETR1 protein. If

TABLE 21-4 Ethylene Binding by the *etr1* mutant^a

Treatment	[^{14}C]Ethylene trapped (dpm/10 g)	
	Wild type	<i>etr</i> mutant
(a) [^{14}C]ethylene ($0.07 \mu\text{l} \cdot \text{liter}^{-1}$)	382 ± 52	135 ± 30
(b) [^{14}C]ethylene ($0.07 \mu\text{l} \cdot \text{liter}^{-1}$) + [^{12}C]ethylene ($667 \mu\text{l} \cdot \text{liter}^{-1}$)	57 ± 10	76 ± 28
Specifically bound (a - b)	325 ± 53	59 ± 41

^aSeven- to 10-g leaves of mutant *etr1* and wild-type plants were incubated in [^{14}C]ethylene plus/minus unlabeled ethylene for 5 h in sealed glass chambers. After incubation, leaves were removed, aired for 50 s, and transferred to a second chamber for 16 h with a beaker containing 1 ml of mercuric perchlorate, which trapped ethylene emanating from the leaves. Radioactivity in the trap was counted. Reprinted with permission from Bleeker *et al.* (1988), © American Association for the Advancement of Science.

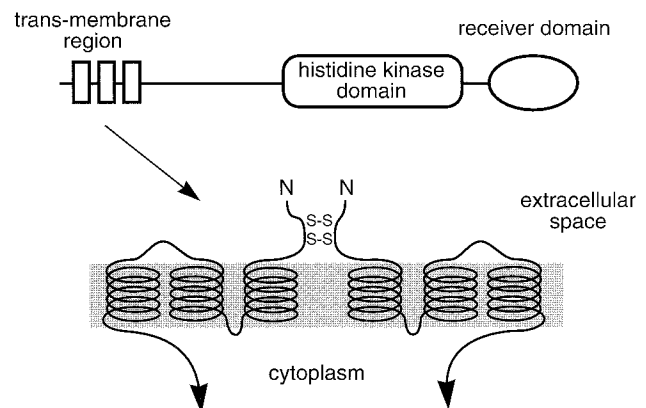


FIGURE 21-13 A schematic illustration of an ETR1 dimer. The two monomers are linked by disulfide bonds. The three transmembrane domains at the N terminus are shown as α -helices. The N terminus itself is outside the membrane. Reprinted with permission from Bleeker *et al.* (1988), © American Association for the Advancement of Science.

mutations are introduced in the transmembrane stretches of *ETR1* by site-directed mutagenesis, and the mutated gene is expressed in yeast, the mutated proteins are unable to bind [^{14}C]ethylene. Such experiments confirm that proper anchoring of the protein in the membrane is important for ethylene binding. The identity of the membrane is not proven, but it is thought to be the plasma membrane.

6.4. Ethylene-Binding Site

While data from transgenic yeast confirm that *ETR1* binds ethylene and functional analysis of the protein, including site-directed mutagenesis, establishes that the N-terminal is important in such binding, a conclusive proof establishing the site of binding and the nature of the transition metal involved was lacking. It has been shown that copper is the transition metal and that Cys⁶⁵ and His⁶⁹ in the second transmembrane domain hold the Cu^{2+} ion in a hydrophobic pocket within the membrane. These results were obtained by a combination of *in vitro*-binding assays using membrane fragments from *ETR1*-transformed yeast cells (Fig. 21-14) and site-directed mutagenesis, which established that Cys⁶⁵ and His⁶⁹ were both crucial for copper binding by membrane fractions. Ethylene is readily soluble in lipids, and entry of ethylene into

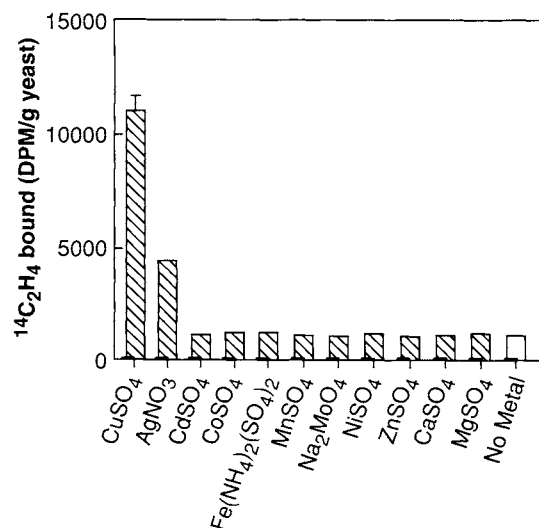


FIGURE 21-14 Effects of transition metals on ethylene-binding activity in yeast membranes expressing the *ETR1* protein. Ethylene-binding assays were performed with assay buffer alone (control) or with $300\ \mu\text{M}$ of the indicated metal salts. Data show clearly that membrane fragments expressing *ETR1* had the highest binding affinity for ethylene in the presence of copper ions, slightly less with silver ions, and hardly any with other transition metals. From Rodriguez *et al.* (1999).

the lipid bilayer of the plasma membrane is not an impediment. An intramembrane site, as shown in Fig. 21-15, would also serve to prevent diffusion of ethylene to the atmosphere and raise its concentration at the active site of the receptor above the required minimum threshold. In confirmation, it was shown

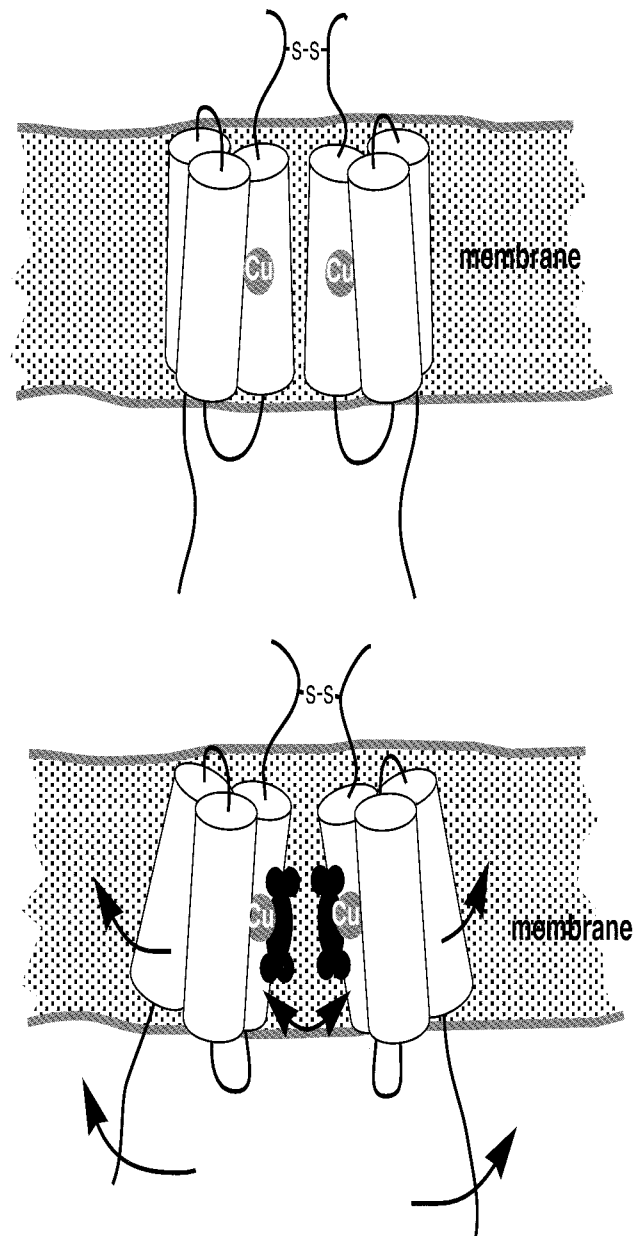


FIGURE 21-15 Schematic illustration of the transmembrane domains of *ETR1* and the binding site for copper. Transmembrane helices are shown as cylinders with copper bound to the second helix. Ethylene (shown as two dumbbells) binding to the copper cofactor is thought to induce a conformational change, which is propagated through the third helix to the histidine kinase transmitter domain. Modified with permission from Bleecker *et al.* (1998), © American Association for the Advancement of Science.

that the half-life of dissociation of ethylene from the binding site within the membrane was ~ 11 h, considerably longer than the dissociation constant of ethylene from metal complexes *in vitro*.

A specific copper-transporting protein RAN1 (a P-type ATPase) in *Arabidopsis*, located in intracellular membrane compartments, is thought to deliver copper ions from intracellular storage sites to Golgi vesicles. In Golgi vesicles, copper is presumably inserted in ETR1-type proteins during their processing in the endomembrane system before transport to the cell exterior.

Taken together, genetic, biochemical, and physiological data provide very convincing evidence that ETR1 is an ethylene receptor.

7. HOMOLOGUES OF ETR1 IN ARABIDOPSIS

Since the initial cloning of the *ETR1* gene, it has become clear that there is a family of ETR1-like pro-

teins in *Arabidopsis*. This family includes EIN4, ETR2, ERS1 (for ethylene response sensor), and ERS2. As shown in Table 21-2, the mutant alleles of *ETR2* and *EIN4* were available from the initial screenings. *ERS1* and *ERS2* were isolated by homology cloning, and mutants *ers1* and *ers2* were created by site-directed mutagenesis. Epistasis analyses using double mutants (e.g., *etr2 ctr1*, *ein4 ctr1*, *ers1 ctr1*) showed clearly that all these proteins act upstream of *CTR1*. The deduced amino acid sequences indicate strong similarities to ETR1 in the N-terminal region, especially in the transmembrane domains, but these proteins also show some specific features (Fig. 21-16A). EIN4 and ETR2 are structurally similar to ETR1 in that they have all three domains, an extracellular region and three putative membrane-spanning domains in the N-terminal half and, in the carboxy half, the histidine kinase and the conserved aspartate in the receiver domain of the response regulator. ERS1 and ERS2 have the N-terminal half and the histidine kinase domain, but they are truncated proteins and lack the receiver

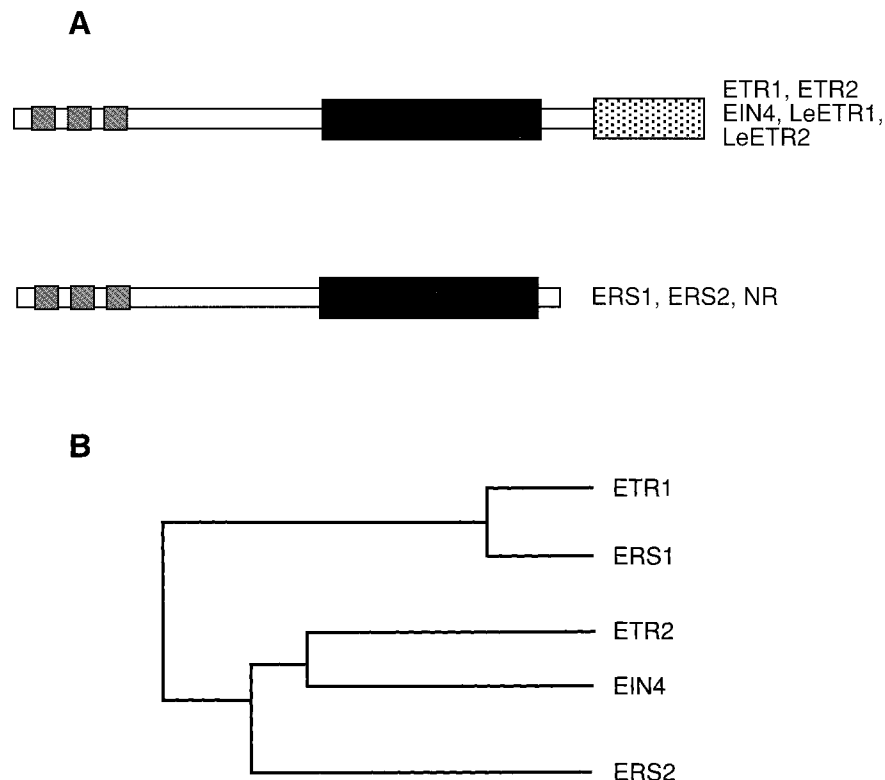


FIGURE 21-16 Schematic illustrations of ETR1 and homologues in *Arabidopsis* and tomato (*Lycopersicon esculentum*). (A) Structures of ETR1, ETR2, and EIN4 in *Arabidopsis* and of ETR1 and ETR2 in tomato contain the transmembrane stretches, the histidine kinase domain, and the receiver domain with the conserved Asp residue. ERS1 and ERS2 in *Arabidopsis* and NR in tomato lack the receiver domain. (B) Based on structural similarity, *Arabidopsis* proteins fall into two subgroups: ETR1 and ERS1 are in one subgroup and the other three are in another group. Adapted from Hua *et al.* (1998).

domain of the response regulator. Based on gene structure, i.e., overall sequence similarity and number and positions of introns, and sequence diversity in the histidine kinase domain, two subfamilies are recognized (Fig. 21-16B): ETR1 and ERS1 are in one subfamily and ETR2, EIN4, and ERS2 are in the second subfamily.

8. INTRAGENIC SUPPRESSORS INDICATE HOW ETR1 AND HOMOLOGOUS PROTEINS ACT

As explained in Appendix 1, a dominant mutation may be a gain-of-function or a loss-of-function mutation and does not permit an easy answer as to how the wild-type gene acts. Since all known mutated alleles of *ETR1* and homologous genes in *Arabidopsis* were dominant mutations, it was not possible to

decide how they acted. This dilemma was solved by creating suppressor mutants of the *etr1* phenotype and specifically screening for intragenic suppressors, as shown schematically in Fig. 21-17.

Several intragenic suppressors were identified for *etr1*, *etr2*, *ein4*, and *ers2*. All were recessive and, therefore, loss of function mutations. Sequencing of the mutant genes (from the suppressor lines) confirmed that the mutations were intragenic and, in most cases, caused by base substitutions, which resulted in premature stop codons and truncated proteins. Since each individual suppressor line displayed a wild-type ethylene response in air and in ethylene (Fig. 21-18A), it was clear that the parental lines of insensitive mutants, *etr1*, *ein4*, etc., were dominant gain-of-function mutations. The loss-of-function mutations provided another important insight into ethylene receptor function. It is intuitively assumed that ligand binding activates a receptor which then turns on the responses. In other words, the receptor acts positively.

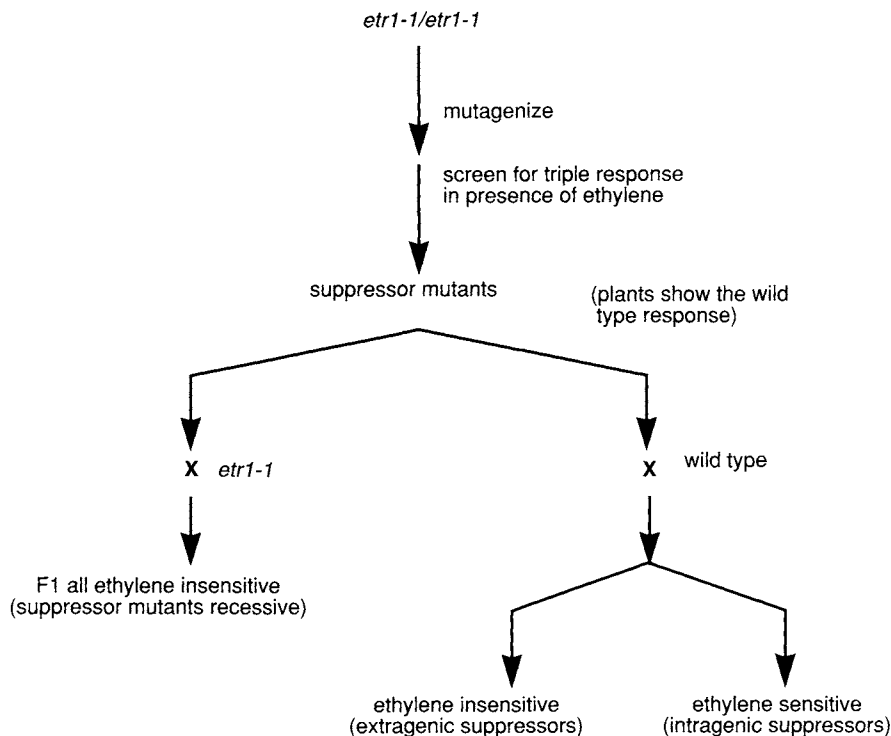


FIGURE 21-17 A scheme showing isolation of intragenic suppressor mutants of the *etr1-1* phenotype. Homozygous plants carrying the *etr1-1* (or *etr1-2*) allele were mutagenized with EMS, and mutants were screened for the triple response by growing them in ethylene. Mutants that showed the triple response, i.e., those that showed a suppression of the *etr1* phenotype, were backcrossed to the parental mutant line (*etr1-1*) to establish whether the mutation was dominant or recessive. Because all F1 plants were ethylene insensitive, it established that the suppressor mutations were recessive. Next, the suppressors were crossed with the wild type to establish whether the suppression arose from mutation in a new gene (extragenic suppression) or whether it resulted from a mutation within the *ETR1* gene (intragenic suppression). Progenies that showed ethylene insensitivity in the triple response were extragenic suppressors; progenies that showed a wild-type ethylene response were intragenic suppressors.

Ethylene receptors, by contrast, are negative regulators of the ethylene responses, i.e., they repress ethylene responses in the absence of ethylene signal. The single loss-of-function receptor mutants show essentially wild-type phenotypes and are indistinguishable from them, which indicates that the genes have redundancy (this explains why only dominant receptor mutations were selected). Some double loss-of-function mutants (*etr1 ein4*), and especially the triple or quadruple loss-of-function mutants display an increasing ethylene sensitivity and ethylene-responsive genes such as *BASIC CHITINASE* are expressed constitutively (Fig. 21-18B). The exaggerated ethylene response phenotype in the triple and quadruple loss-of-function receptor mutants is not due to overproduction of ethylene, because the use of ethylene biosynthesis inhibitor aminoethoxyvinylglycine has no effect. Since the loss of multiple receptors results in consti-

tutive ethylene responses, the wild type receptors must actively repress ethylene responses in air (or in the absence of ethylene signal).

9. ETHYLENE SIGNALING PATHWAY IN *ARABIDOPSIS*

Knowledge of the key components in ethylene signaling in *Arabidopsis*, and the order in which they act, makes it possible to propose a model for ethylene signaling. In this model, CTR1 and EIN2 play central roles. CTR1 receives the signal from upstream elements, such as ETR1, and passes the signal downstream to EIN2. Since recessive mutations in CTR1 result in a constitutive ethylene response, the CTR1 protein acts as a negative regulator of the ethylene response path

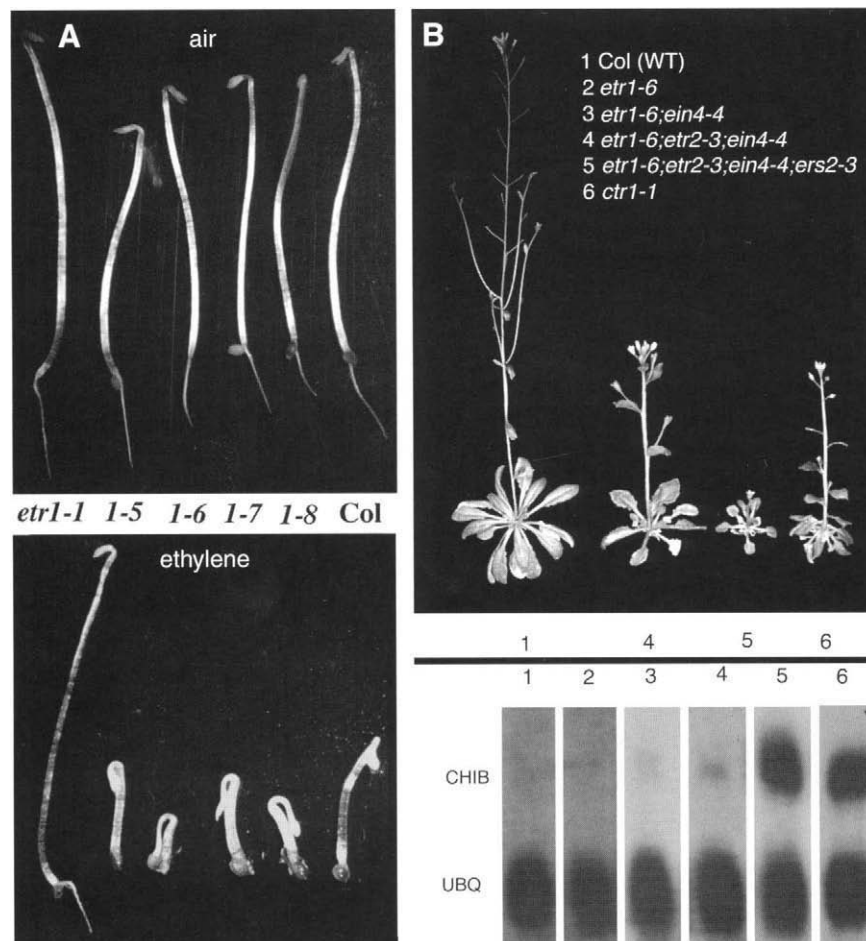


FIGURE 21-18 Phenotypes of four intragenic suppressors of an ethylene-insensitive mutant in air and in ethylene. Designations are *etr1-1*, an ethylene-insensitive mutant; *etr1-5*, the intragenic suppressor line for *etr1-2*; and *etr1-6*, *1-7*, and *1-8* are suppressor lines for *etr1-1*. Col, wild-type *Arabidopsis* ecotype Columbia. Reprinted with permission from Hua and Meyerowitz (1998), © Cell Press.

way. The structural similarity of CTR1 to Raf-1 type MAPKKK suggests that CTR1 signals to EIN2 via a MAPK cascade, but to date no evidence for such signaling has been obtained. The response pathway from EIN2 downstream is turned ON, if CTR1 protein is inactivated either due to a mutation in CTR1 or because of a signal from an upstream element, such as, ethylene binding to ETR1 type receptors. A wild-type EIN2 is essential for receiving the signal from CTR1 and activation of ethylene response pathway because *ein2* mutations result in an ethylene-insensitive phenotype. EIN2 also seems to be a junction point for further downstream ethylene signaling and cross-talk with signaling pathways for other hormones, such as IAA, cytokinins, ABA, and jasmonates.

The presence of many homologues of ETR1 suggests that ethylene perception in *Arabidopsis* involves multiple receptors or a receptor complex, certainly ETR1 and likely ERS1, ETR2, EIN4, and ERS2 as well (see Section 11). Among these, ERS1 has also been shown to bind ethylene using *ERS1*-transformed yeast cells and a binding assay similar to that used for ETR1 (see Fig. 21-12). The information gained from intragenic suppressors of *etr1-1*, *etr2-1*, *ein4-1*, and so on allows us to conclude that the receptor (or receptor complex), in air, keeps ethylene responses actively repressed. Thus, the ETR1 protein is thought to be autophosphorylated in the absence of ethylene, and an activated CTR1 keeps the ethylene response pathway inhibited. In the presence of ethylene, ETR1 is not phosphorylated, CTR1 stimulation is turned off, and downstream ethylene signaling begins. A dominant mutation in a receptor protein, such as ETR1, locks ETR1 (or the receptor complex) in an activated state, such that CTR1 is constantly turned on, inhibiting downstream ethylene signaling (Fig. 21-19). A mutation in the gene encoding the copper-transporting protein also gives a constitutive ethylene response

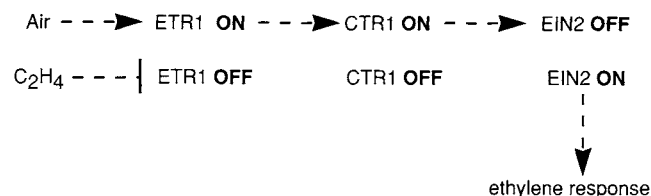


FIGURE 21-19 A scheme for ethylene signaling. In the absence of ethylene, ETR1 is autophosphorylated, it activates CTR1, and an activated CTR1 inhibits the ethylene response pathway from EIN2 onward (it would seem that EIN2 is inactivated when phosphorylated, but this needs to be confirmed). In the presence of ethylene, ETR1 is not phosphorylated, there is no signal to CTR1, and EIN2 is on and activates the ethylene responses. ETR1 homologues, ETR2, EIN4, ERS1, and ERS2, could act in a complex with ETR1.

phenotype because it seemingly disables the receptor or receptor complex.

10. ETHYLENE SIGNALING IN OTHER PLANTS

Homologues of ETR1 have also been identified in some other plants, notably tomato (*Lycopersicon esculentum*). The advantage of working with tomato is that, in addition to the triple response of seedlings, some other ethylene responses, such as fruit ripening and senescence, can be studied. Five receptor genes have been identified in tomato, *LeETR1* (for *Lycopersicon esculentum* ETR), *LeETR2*, *LeETR3* better known as *NR*, *LeETR4* and *LeETR5*. The *never ripe* (*nr*) mutant of tomato does not show ripening of fruit and is ethylene insensitive. The *nr* allele is inherited as a single dominant trait and, like the *etr1*, is pleiotropic in its effects, blocking fruit ripening, senescence and abscission of flowers, and epinastic response of petioles. The *NR* gene as well as *LeETR1* and *LeETR2* encode proteins similar to ETR1, except that *NR* is a truncated protein and lacks the receiver domain of the response regulator (see Fig. 21-16). *LeETR4* and *LeETR5* also encode full length proteins, but show much less amino acid sequence identity (only about 40% vs about 80% for *LeETR1* and *LeETR2*). Although the amino acids known to be involved in ethylene binding are conserved in *LeETR4* and *LeETR5*, these proteins lack several conserved amino acids within the histidine kinase domain that are predicted to be involved in phosphorylation. Since mutated forms of both *LeETR4* and *LeETR5* confer dominant ethylene insensitivity in transgenic *Arabidopsis* plants, these data appear to indicate that histidine kinase activity is not necessary for an ethylene response.

Although most work on ethylene signaling has been done to date using *Arabidopsis* and tomato, some homologous clones are beginning to be isolated in other plants as well (e.g., tobacco, muskmelon, carnation). While it is premature to conclude that ethylene signaling as postulated for *Arabidopsis* occurs universally in plants, the presence of ETR1 and ERS homologues in tomato encourages the idea that it proceeds in a similar manner in other plants as well.

11. WHY ARE THERE SO MANY HOMOLOGUES OF A "RECEPTOR" PROTEIN?

The presence of multiple genes encoding full-length or partial receptor-like proteins in *Arabidopsis* and

tomato raises the question of why are there so many potential receptor candidates in a single plant? The answer is unclear, although there are several possibilities.

i. Modulation of signal. Dimerization of ETR1 was referred to in Section 6.2. The presence of truncated proteins such as ERS1 and ERS2 and NR highlights a common theme among homologues of regulatory proteins. Similar truncated, but selectively functional proteins are known from some receptor-like protein kinases involved in self-incompatibility or morphogenetic responses in plants (see Chapter 25) and, as mentioned in Appendix 1, for transcription factors of the basic region leucine zipper (bZIP) and basic region helix-loop-helix (bHLH) classes. The presence of truncated proteins and their ability to form heterodimers are believed to allow modulation of the signal. ERS1, and presumably ERS2 and NR, proteins can bind ethylene and thus participate in signal perception and its amplification, but cannot signal downstream on their own.

ii. Tissue/organ specificity of response. It is also possible that the various receptor candidates operate in a tissue-specific and/or response-specific manner, although available data are not sufficient to provide a clear conclusion. Expression of both *Arabidopsis* and tomato genes has been studied developmentally as well as in response to ethylene. In *Arabidopsis*, mRNAs of all five genes were expressed at low levels ubiquitously in most organs and tissues. However, the accumulation of *ETR1* and *EIN4* mRNAs was constitutive and independent of ethylene treatment, whereas the accumulation of *ETR2*, *ERS1*, and *ERS2* mRNAs was dependent on ethylene treatment. Similarly, in tomato, both *LeETR1* and *LeETR2* mRNAs were constitutively expressed in all organs, although there were some quantitative differences between the two, whereas *NR* mRNA was expressed selectively in developing ovaries and ripening fruit and was ethylene dependent. Thus, it appears that while some proteins of the receptor family in a species are constitutively expressed, the expression of others is more precisely regulated by the hormone itself.

iii. Functional compensation. The presence of multiple receptors provides the plant with redundancy and a measure of security. This means that the loss of function of one receptor may be compensated for by the presence, or enhanced expression, of other functional receptors. A compensatory mechanism ensures that the loss of one (or two) receptors does not disable the plant from the protective action of ethylene against environmental stresses, nor does it prevent normal growth in air. As mentioned in Section 8, the isolation

of the loss-of-function receptor mutants in *Arabidopsis* revealed this phenomenon in a striking manner. Since ethylene receptors are thought to actively repress ethylene responses in air, the loss of receptor action correlates with the loss in repressive activity and enhanced ethylene sensitivity. Assuming that *ETR1*, *ETR2*, *EIN4*, *ERS1*, and *ERS2* are all receptor proteins, the loss-of-function of one, or even two, receptor proteins still leaves the plant with a fairly normal response in air or in ethylene. However, if three or more receptor proteins lose function simultaneously, then the compensatory mechanism fails and plants show an exaggerated ethylene sensitivity, even constitutive ethylene responses (Fig. 21-20). Loss-of-function of a receptor protein can be duplicated by reduced amounts of the receptor protein using antisense technique. Thus, in tomato, expression of *LeETR4* in an antisense orientation in transgenic plants resulted in extreme ethylene sensitivity. Multiple responses were affected, including severe epinasty, enhanced flower senescence, and accelerated fruit ripening. These data from tomato may appear to be at variance from those of *Arabidopsis* where at least two receptors must lose function for enhanced ethylene sensitivity; but, more interestingly, the effects of reduced levels of *LeETR4* protein could be countered by overexpression of *NR* gene indicating a functional compensation between the two receptor genes. By contrast, transgenic tomato plants expressing the *NR* gene in an antisense orientation display a normal phenotype, which suggests that other *LeETRs* are able to compensate for reduced levels of the *NR* protein. These data from tomato complement those from *Arabidopsis*; (they are indicative of functional compensation between the receptor proteins, they also suggest functional differences between them.

iv. Extending the range and/or "filtering" of signal. Still another possibility is that receptors with varying affinities for ethylene provide a range of ethylene concentrations over which plants can respond to ethylene. Some data for kinetics of dissociation of ethylene from the receptor site already exist and indicate that the half-life of dissociation may vary from ~30 min to about 6 h. Along the same lines, it is also possible that in a complex of receptor-like proteins, some serve as "decoys" to screen out the spurious ligands and leave only the "true" ligands for the "true" receptors. In the natural environment of plants, there are hundreds of possible ligands that may crowd the binding sites. Thus, it would be helpful if there were an excess of receptors that are more catholic and bind a large number of ligands with low affinity but higher volume, and thus are able to "filter" the true ligands (in this case, ethylene, which occurs in low concentration) for binding to *ETR1* or *LeETR1*.

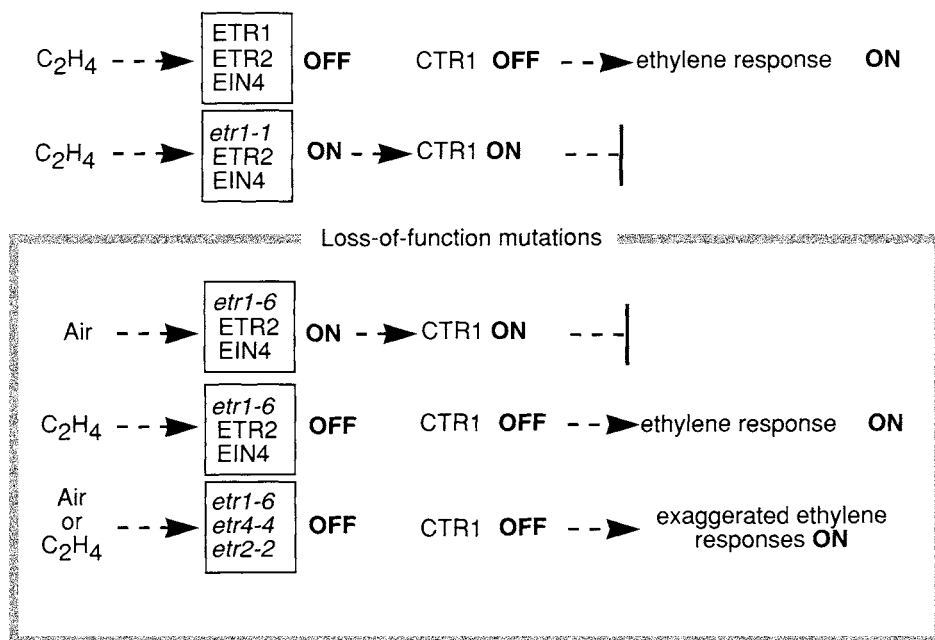


FIGURE 21-20 A schematic illustration of the ethylene-induced response in the wild type, an ethylene-insensitive dominant mutant, *etr1-1*, and in loss-of-function mutants of *ETR1* or homologues (boxed lower part). The wild type and the ethylene-insensitive dominant mutant (*etr1*) show a normal response to ethylene. In the boxed area, if one receptor loses function, the response in air or in ethylene is still normal. However, if three (or four) receptors lose their function simultaneously, ethylene responses are exaggerated.

12. INHIBITORS OF ETHYLENE RESPONSE

Silver ions (used as silver thiosulfate) and organic compounds, such as 2,5-norbornadiene, *trans*-cyclooctene, and 1-methylcyclopropene, are potent inhibitors of ethylene-induced responses (see Chapter 11, Section 8). The mechanism of inhibition is not known. Since ethylene binding by yeast membranes expressing ETR1 is enhanced by coincubation with silver salt (see Fig. 21-14), it may be that silver ions inhibit ethylene action by replacing copper in the ethylene-binding sites of the ethylene receptors. The receptor can still bind ethylene but is no longer able to transduce the signal. The inhibition of ethylene action by organic compounds is probably competitive because it requires continuous exposure to these compounds.

13. SUMMARY AND CONCLUSIONS

Ethylene mediates many events in plant growth and development, including seed germination and seedling growth, fruit ripening, and senescence and

abscission. It also mediates many stress-related responses due to pathogens or wounding.

Molecular genetic studies using response mutants in *Arabidopsis* indicate that ethylene is perceived at the cell surface by a family of receptors, which probably act as dimers and which bind ethylene *via* a copper-containing site in the receptor located within the membrane. The signaling pathway proceeds *via* a phospho transfer cascade involving several proteins, but the details of phospho-transfer and proteins involved are still unknown. According to one hypothesis, the ethylene receptor ETR1 is autophosphorylated in the air, the signal is transmitted to CTR1 which *via* a MAP kinase cascade (or an alternate pathway) keeps the ethylene response pathway repressed. In the presence of ethylene, ETR1 is not autophosphorylated, CTR1 does not receive a signal, and the downstream ethylene response pathway via EIN2 is turned on leading to activation of transcription factors in a tissue- and response-specific manner. Some transcription factors such as EIN3, and ethylene response factor1 (ERF1) and similar proteins (EIL) have been identified. EIN3 activates the transcription of *ERF1* gene, and the ERF1 protein, in turn, activates the transcription of pathogenesis-related *BASIC CHITINASE* genes. A similar mechanism may be responsible for transcription of other ethylene-responsive genes.

EIN2, an intermediate between CTR1 and further downstream elements (e.g., EIN3/EIL1), seems to be a multifunctional protein where ethylene signaling interconnects to signaling pathways from other hormones.

The presence of proteins similar to ETR1 in *Arabidopsis* and tomato suggests that the receptors for ethylene occur in multimeric complexes. Although only ETR1 and ERS1 in *Arabidopsis* have so far been shown to bind radiolabeled ethylene, it is a strong possibility that others are able to do so. That receptor proteins may function as a complex confers several advantages to the plant: it can extend the range of concentration over which ethylene may be perceived; some proteins may serve as decoys and filter out the ethylene signal from other signals; it provides needed redundancy such that even if one or two proteins lose their function, the complex can nonetheless still sense ethylene and trigger ethylene-mediated defense responses; some receptor proteins may be selectively active in certain tissues/organs; and the ethylene signal may be enhanced or attenuated by the formation of homo- or heterodimers.

There are still some gaps in our knowledge of the signaling pathway. For instance, the immediate downstream elements from ETR1 (or its homologues), the immediate elements on either side of CTR1, and the connecting links between EIN2 and EIN3/EIL proteins are unknown. Although phospho-group transfer is implied in signaling from histidine kinase domain of ETR1, via the kinase domain of CTR1 downstream to EIN2, the details of such transfer including the existence of a MAPK cascade if any, are obscure. The precise manner in which the ethylene signaling cross-talks with signaling pathways from other hormones is also unknown. Nonetheless, information gained from *Arabidopsis* and tomato has been a signal achievement in the perception of a plant hormone and transmission of the signal to the point of gene expression. It has opened the way for research on other plants and a verification whether the pathway as deduced from *Arabidopsis* is universal in plants.

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1. AUXIN-INDUCED RESPONSES

As covered elsewhere, auxins are involved in various developmental and physiological responses (see Chapters 14, 15, 17, 20, and 27). They regulate apical-basal polarity in embryo development, apical dominance in shoots, induction of lateral and adventitious roots, vascular tissue differentiation, and cell growth in stems and coleoptiles, including asymmetric growth associated with phototropic and gravitropic curvatures. Because of their obvious importance in plant growth and development, an immense amount of research has gone into elucidating the mechanism of auxin action. This research, spanning over three

decades, has made important contributions to our understanding of the molecular biology of auxin action and is the subject of this chapter.

2. AUXIN-BINDING PROTEINS

Much of the early work in the 1970s and 1980s on the molecular basis of auxin action was devoted to characterizing auxin-binding sites in different plant tissues (for methods, see Appendix 4). These included systems for cell elongation (e.g., corn coleoptiles, pea or bean epicotyl/hypocotyl segments) and cell division (e.g., dividing cells of tobacco pith callus cultures). The assays used radiolabeled IAA and synthetic auxins (1-NAA, and 2,4-D), as well as phytochemicals that block polar auxin transport, such as naphthylphthalamic acid (NPA). These studies revealed several binding sites, which satisfied the criteria for receptor binding, i.e., specificity, exchangeability, and high affinity.

Three membrane-bound sites were identified. site I, microsomal fraction, mostly endoplasmic reticulum (ER); site II, Golgi bodies and/or tonoplast; and site III, membrane fraction mostly from plasma membrane (PM).

The identification of membranes was based on density in a sucrose gradient and presence of membrane-specific enzyme markers (e.g., NADPH-dependent cytochrome C reductase for ER; cytochrome C oxidase or concanavalin A binding for PM; glucan synthetase for Golgi). Other binding sites were soluble, i.e., binding was obtained using the super-

natant after centrifugation of the plant extract at $100,000 \times g$.

2.1. Membrane-Bound Sites

It was clear from the beginning that sites I and III were different. Site I, associated with the ER membranes, bound the auxins, IAA and NAA, with high affinity, and NPA little or not at all. In contrast, site III, associated with the plasma membrane, bound NPA with much greater affinity than IAA or NAA. Site III proved to be the auxin-efflux carrier involved in the polar transport of auxin, as discussed in Chapter 13. It is not considered further in this chapter.

Site II has never been distinguished unambiguously from site I and there is no evidence that it represents a different binding protein.

Site I binding has been pursued extensively, and many researchers believe that it represents binding to an auxin receptor. Early hormone-binding assays showed that ER fractions from maize coleoptiles bound radiolabeled 1-NAA specifically and with high affinity (Fig. 22-1). Binding was due to a protein, which could be separated easily from membrane frac-

tions by acetone or detergent treatment and still show *in vitro* binding.

Moreover, the binding was physiologically significant. It occurred over the same range of auxin concentrations that brought about elongation of coleoptile segments. It had a pH optimum of about 5.5 (Fig. 22-2), which agreed with the pH optimum of auxin-mediated coleoptile growth. Maize mesocotyl elongation is inhibited by red light. It was shown that illumination of maize seedlings with red light also abolished the site I binding activity. Auxin analogs competed for the binding site in proportion to their biological activity, whereas nonactive analogs (e.g., indolepropionic acid, benzoic acid, 2-naphthalene acetic acid) competed less well, and other hormones, such as gibberellin A₃ and abscisic acid, did not compete at all. Curiously, 2,4-D, another synthetic auxin, was relatively ineffective in displacing bound 1-NAA or IAA.

2.1.1. Site I Protein from Maize

The site I protein was purified to homogeneity. It is a 22-kDa protein, which occurs as a dimer in natural state with a molecular mass of about 45 kDa. Its cDNA

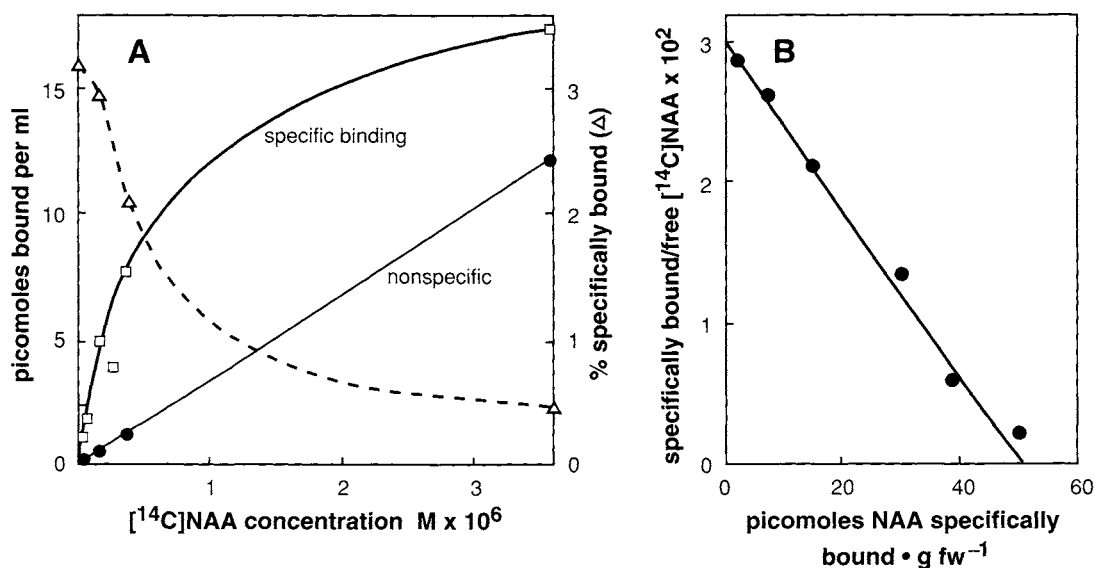


FIGURE 22-1 (A) Specific (\square) and nonspecific (\bullet) binding of [^{14}C]NAA to membrane fractions from maize coleoptiles. Membrane fractions were incubated with [^{14}C]NAA (at $5 \times 10^{-8} \text{ M}$) plus nonradioactive NAA at varying concentrations (from 0 to saturating concentration of 10^{-4} M) to yield the curve for specific binding. Note that specific binding of [^{14}C]NAA rises steeply at first, and then plateaus as the binding sites get saturated. Note also that the [^{14}C]NAA specifically-bound as a percentage of that totally bound (\triangle) decreases as the total hormonal concentration is increased. This is because nonspecific sites also begin to bind the hormone. Data for nonspecific binding have been divided by 10 for plotting so that the large value obtained at the highest [^{14}C]NAA concentration could be included on the scale. (B) Scatchard plot for specifically bound NAA. For this plot, [^{14}C]NAA specifically bound in moles and the amount of free radioactivity in molar concentrations were calculated and plotted against [^{14}C]NAA specifically bound in moles per g^{-1} fresh weight. From Ray *et al.* (1977).

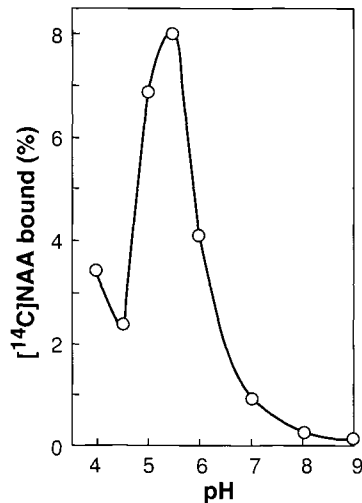


FIGURE 22-2 pH dependence of auxin binding. An ER-enriched fraction from maize coleoptiles was divided into several equal fractions kept in the same buffer. Each fraction was adjusted to a different pH and used for specific binding assays using [³H]NAA, plus/minus an excess of unlabeled NAA. From Ray *et al.* (1977).

and gene have been cloned, and homologous cDNAs from several other plants, both monocots and dicots, are available. The deduced amino acid sequences point to a protein of 20–22 kDa in different species. Genomic analysis indicates that these proteins are encoded by members of small gene families (e.g., five members in maize, six in strawberry, two in tobacco, one in *Arabidopsis*). Northern hybridizations using gene-specific cDNA probes suggest that different isoforms of the protein are expressed in different organs in a development-specific manner.

The maize protein is also detected using photoaffinity-labeled auxins. The photoaffinity probes (see Appendix 4) have yielded many other proteins that bind auxins. Hence, to distinguish it from other proteins, the 22-kDa protein from maize and its homologues in other species are referred to as auxin-binding protein 1 (ABP1).

ABP1 is a very well-investigated protein in plant hormone research. Many investigators believe that it represents an auxin receptor, whereas others remain skeptical, partly because it shows several unusual features in structure, cellular location, and relative abundance in different plants. In the following, evidence both for and against ABP1 being an auxin receptor is discussed. Although ABP1 proteins have been identified by immunostaining and/or Western blots in all plant species examined, in most species they are present in much smaller quantities than in maize. As a result, the protein that has been studied in detail is the ABP1 from maize (*Zea mays*, Zm-ABP1).

2.1.2. Structure and Functional Significance of ABP1

2.1.2.1. Cellular Location of ABP1

All ABP1 sequences identified to date have an N-terminal signal peptide that targets the protein for entry into the endoplasmic reticulum and, which on entry, is cleaved off. They also have the sequence of four amino acids, -Lys -Asp -Glu -Leu- (the KDEL sequence), at the C terminus, which is specific for proteins that are retained within the lumen of the endoplasmic reticulum (Fig. 22-3) (see also Appendix 3). Sequence comparisons with proteins in databases have revealed no significant homologies.

That ABP1 is resident in ER is confirmed by several other observations. ABP1 proteins, like many other proteins, are glycosylated, i.e., they carry short oligosaccharide chains (one or two), but in this case, the chains are rich in mannose residues, a feature typical of ER resident proteins. The ABP1 protein has been expressed transgenically in heterologous hosts, including plants, insect, and mammalian cells. In all cases, it has been shown to be resident in the ER. Immunostaining of microsomal preparations or *in situ* immunofluorescence studies using antibodies against the protein also indicate that the protein is localized in the ER. While these observations explain data from earlier binding assays that associated site I binding with the endoplasmic reticulum, other experiments point to the auxin signal being perceived on the cell surface. In the following, evidence for and against ABP1 being an auxin receptor is presented.

2.1.2.2. Evidence That ABP1 Is an Auxin Receptor

In experiments on the functional significance of ABP1, polyclonal as well as monoclonal antibodies, prepared against the whole protein or against synthetic peptides based on specific sequences in the protein, have played a very important role. A linear map of the ABP1 from maize representing these specific regions (epitopes) and the monoclonal antibodies against them is shown in Fig. 22-4. The map also shows two regions in the protein, box D16 and two conserved amino acid residues, Asp (D) and Trb (W) (marked by a large open arrow), which are thought to act as the binding sites for auxins, such as IAA and 1-NAA.

A receptor protein, by definition, is involved in perceiving the hormonal signal and either negatively or positively regulating the chain of events, or steps, that lead ultimately to an overt physiological or biochemical response. The earlier an event can be detected, the closer it is likely to be to the initiating molecule, i.e., the receptor. Among the earliest responses associated with auxin-induced cell elongation

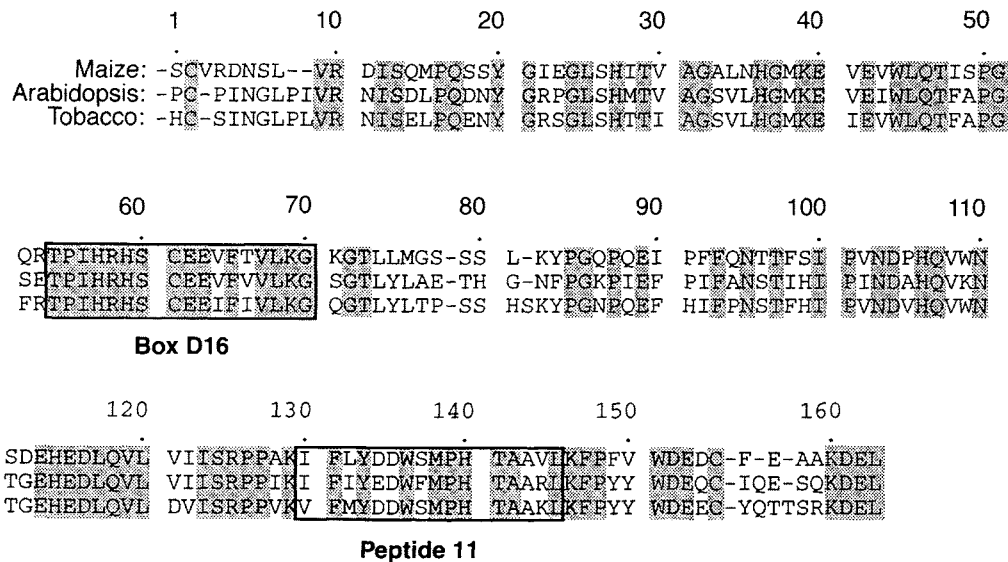


FIGURE 22-3 Amino acid sequence comparisons of auxin-binding protein 1 (ABP1) from maize, *Arabidopsis*, and tobacco. Sequences were deduced from cDNAs and are repositioned for maximal alignment. Identical residues are shaded. The first 38 residues (in maize) comprise the signal peptide for entry into the endoplasmic reticulum, whereas the last 4 residues, KDEL, are the ER retention signal. The significance of the two boxes, box D16 and peptide 11, is explained later. Amino acid numbers are indicated above the sequences. Modified from Brown and Jones (1994).

are a hyperpolarization of the plasma membrane, which is correlated with the induction of H^+ -ATPases, and induction of primary auxin response genes. Both are triggered within 5–15 min of exogenous hormone being supplied (see Chapter 15 and Section 3.1. below).

The steady-state transmembrane potential (E_m) of plasma membrane in intact cells of coleoptile or stem segments is usually between -100 and -130 mV. Treatment of these segments with auxin induces a biphasic response, an initial depolarization, followed about 7–12 min later by a steep hyperpolarization (about 25 mV more negative than the steady state) (Fig. 22-5A). To study the effects of antibodies against *Zm*-ABP1 on the hyperpolarization response, however, intact cells/tissues cannot be used because cell

walls, although permeable to small molecules like auxin, are impermeable to larger molecules, such as proteins above 100 kDa. Thus, to study effects of antibodies on the hyperpolarization response requires the use of protoplasts. Protoplasts show hyperpolarization when incubated with auxins, but there are some marked differences between the auxin-induced hyperpolarization response of intact cells vs that of protoplasts. The steady-state transmembrane potential of plasma membrane in protoplasts is slightly negative (Fig. 22-5B, closed symbols). On addition of auxin, there is no depolarization and hyperpolarization is extremely rapid, it occurs within 2 min of exposure to auxin, and is much shallower (about -6 to -15 mV) (Fig. 22-5B, open symbols; see also Figs. 22-5C and

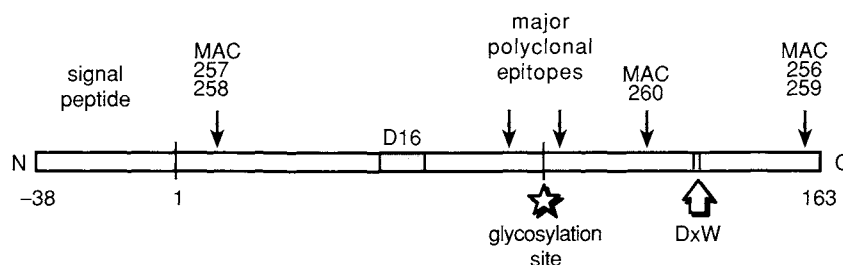


FIGURE 22-4 Linear representation of maize auxin-binding protein 1. Antibody-binding sites are indicated above the protein. MAC numbers are the names of monoclonal antibodies. The D16 peptide sequence, which may be associated with the binding of the carboxyl group, and the residues, aspartic acid (D)¹³⁴ or tryptophan (W)¹³⁶, believed to be closest to the indole or naphthalene ring of auxins, are indicated. Reprinted with permission from Napier (1995), © Oxford University Press.

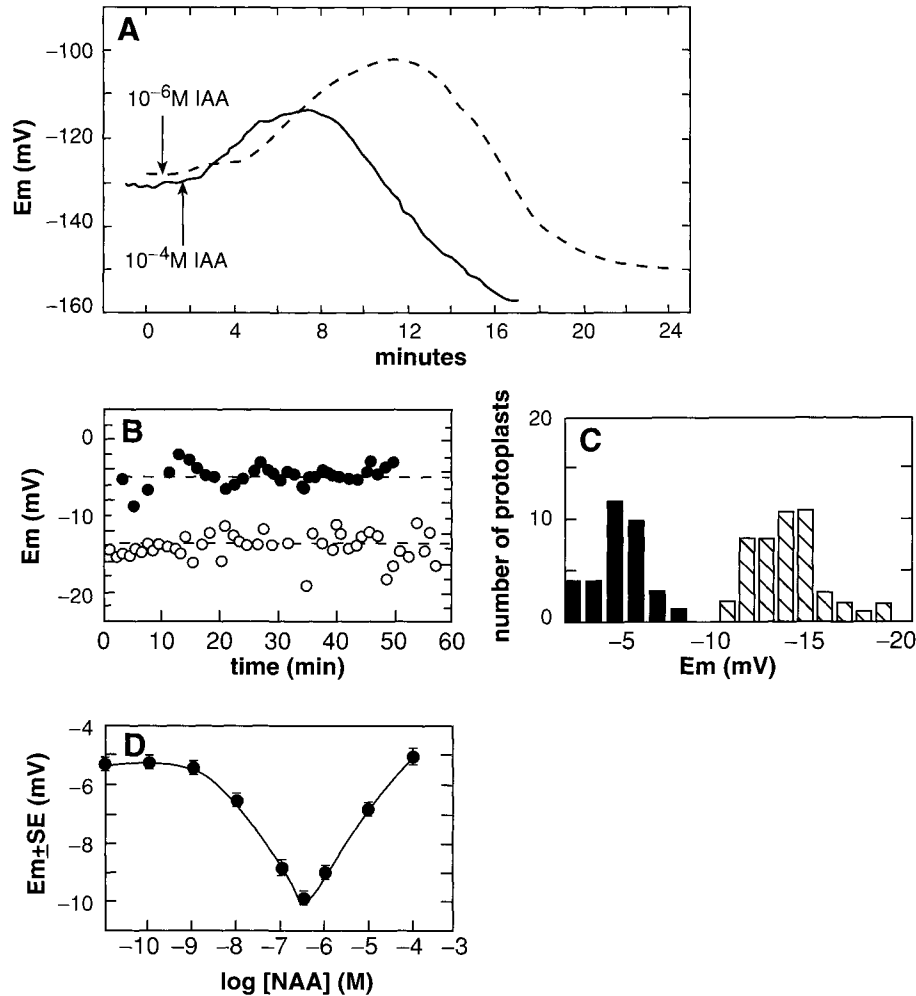


FIGURE 22-5 Hyperpolarization responses of coleoptile segments and tobacco leaf protoplasts. (A) Oat coleoptile segments were split lengthwise and kept in a low salt buffer. A microelectrode (glass capillary filled with 3 M KCl solution) was inserted in a subepidermal cell while being viewed under a microscope, while the reference electrode was in the buffer. After the steady state was reached, IAA was added to the medium (arrows) and changes in transmembrane potential were recorded. Reprinted with permission from Bates and Goldsmith (1983). (B) Evolution of E_m values in a protoplast suspension isolated from tobacco leaf mesophyll in the absence (●) or presence (○) of $5 \mu\text{M}$ 1-NAA added at time zero. Each dot represents an individual measurement on a single protoplast. (C) Data from individual protoplasts in B are replotted as distribution histograms of E_m values. Hatched bars, treated with NAA; closed bars, not treated. (D) Dose-response curve to 1-NAA of the membrane potential of tobacco mesophyll protoplasts. Each dot represents an average E_m value from 20 individual measurements. Standard errors are indicated. Reprinted with permission from Barbier-Brygoo (1995).

22-5D). Despite these differences, tobacco mesophyll protoplasts offer a convenient system and have been used extensively to study the auxin-induced hyperpolarization response.

i. Antibodies against *Zm*-ABP1 inhibit the hyperpolarization response of protoplasts. The first clear evidence that *Zm*-ABP1 had a role in auxin-induced hyperpolarization came with the demonstration that antibodies prepared against *Zm*-ABP1 inhibited the hyperpolarization response of tobacco leaf mesophyll

protoplasts (Table 22-1). The response was inhibited increasingly with increasing concentration of the antibody and this correlation could be used to deduce the concentration of putative receptor (ABP1) molecules on the protoplast surface. Addition of *Zm*-ABP1 to the incubation medium enhanced the sensitivity of protoplasts to exogenous NAA, implying an increase in auxin receptor concentration on the cell surface.

Inhibition of the hyperpolarization response of protoplasts by anti-ABP1 IgG suggests that ABP1 has

TABLE 22-1 Effect of 1-NAA and Antibodies against *Zm*-ABP1 (Anti-ABP1 IgG) on the Transmembrane Potential (Em) of Tobacco Leaf Mesophyll Protoplasts^a

Addition to medium	Em (mV)	NAA induced (Δ mV)
None	-5.3 ± 0.3	—
NAA (5 μ M)	-12.7 ± 0.3	-7.4
NAA (5 μ M) + anti-ABP1 IgG (4 nM)	-11.2 ± 0.4	-5.9
NAA (5 μ M) + anti-ABP1 IgG (40 nM)	-8.6 ± 0.4	-3.3
NAA (5 μ M) + anti-ABP1 IgG (400 nM)	-6.0 ± 0.4	-0.7
None	-5.6 ± 0.2	—
Anti-ABP1 IgG (400 nM)	-5.8 ± 0.2	—

^aEm measurements were done using individual protoplasts while they were immobilized in a holder in the culture medium, in the absence (control) or in the presence of 5 μ M NAA. Aliquots from the same protoplast suspension were preincubated with various concentrations of anti-ABP1 IgG (4 to 400 nM) for 2.5 h at room temperature, and NAA was added to 5 μ M. In an independent experiment, the effect of anti-ABP1 IgG at 0.4 μ M alone was tested on Em. Em values are presented as mean \pm standard error calculated for 17–40 individual measurements in each condition. From Barbier-Brygoo *et al.* (1989).

a role in auxin perception and that it is localized on the external face of the plasmalemma. Several other experiments support these conclusions.

ii. Impermeable auxin conjugates can bring about auxin responses. Synthetic auxin molecules that were rendered impermeable across the plasma membrane by coupling them to large proteins or Sepharose beads [e.g., 5-aminonaphthalene-1-acetic acid (5-amino-NAA) conjugated to bovine serum albumin, or to Epoxy Sepharose] were used to check the hyperpolarization response in tobacco mesophyll protoplasts, as well as to induce an elongation response in pea epicotyl segments where the epidermal cuticle had been abraded to facilitate entry of the conjugates across the cell wall. In both cases a positive response was obtained.

iii. An antibody against a conserved region of ABP1 elicits the hyperpolarization response. A synthetic peptide (14 amino acids) was prepared using the amino acids that were predicted to form the auxin-binding pocket in the receptor protein. This synthetic peptide, conjugated to another protein, was used as an antigen to produce antisera. Immunoglobulins purified from one of the antisera, D16, were shown by Western blots to recognize ABP1 isoforms and homologues from a variety of plants, maize, barnyard grass, mung bean, and pea. Such recognition suggested that the peptide sequence against which D16 had been raised was highly conserved, a prediction that is borne out by a comparison of amino acid sequences of ABP1s, deduced from cDNA clones from 11 species of monocots and dicots (box D16 in Fig. 22-3 shows only 3 species).

It was argued that if this peptide represented the auxin-binding site, antibodies against it should be able

to bring about an auxin-induced response in the absence of auxin. The D16 antibody was indeed shown to bring about a hyperpolarization response in a dose-dependent manner in protoplasts from tobacco leaves and maize coleoptiles in the absence of auxin. Thus, whereas antibodies against the whole protein inhibit auxin action, i.e., they act as auxin antagonists, antibodies against a small specific region act as auxin agonists and bring about the same response as auxins.

iv. Azido IAA binds to a peptide from ABP1. To further dissect the region in ABP1 that binds auxins, *Zm*-ABP1 was labeled with [³H]azido IAA, completely digested by trypsin, and the peptide fragments separated by HPLC. One fragment, peptide 11, was bound photolytically to tritiated 5-N₃IAA, and the binding was shown to be specific in that it was eliminated by preincubation with 50 μ M IAA. The peptide fragment is shown in Fig. 22-3 and as D \times W in Fig. 22-4.

v. The C terminus of ABP1 may have a role in signal transduction. The antibody MAC256 binds to the C terminus of *Zm*-ABP1 (see Fig. 22-4). It fails to do so in the presence of physiologically active auxins, presumably because of a conformational change in the protein such that the C terminus is no longer available for binding to MAC256. Accordingly, a 14 amino acid synthetic peptide comprising a portion of the C terminus of ABP1 was used to study the stomatal response in *Vicia faba* guard cells. Low concentrations of auxin are known to bring about stomatal opening, whereas high concentrations (30–100 μ M) are inhibitory. Treatment of epidermal peels with this peptide rapidly (within 30 s) and reversibly inactivated the inwardly directed K⁺ channel, causing closure of stomata (see Section 3, Chapter 23).

vi. Plants transformed with ABP1 show auxin-mediated cell enlargement. In recent years, molecular techniques have been used to provide a more direct demonstration of the role of ABP1 in auxin signaling, though not necessarily as a receptor. An *Arabidopsis* cDNA encoding ABP1 was used to transform tobacco plants under a promoter that could be induced in a controlled manner by supplying the plants with an analog of tetracycline. Induction of ABP1 in the transformed tobacco plants resulted in leaves with normal morphology, but significantly larger cells. In a parallel experiment, maize plants were transformed with the same cDNA clone but under a constitutive promoter. Endosperm cells in maize kernels normally do not show an auxin response, but in the transformed maize plants they were induced by exogenous auxin and were much larger in size. These data linked cell enlargement with ABP1 expression. In another approach, reverse genetics was used to obtain mutant alleles of *ABP1* in *Arabidopsis*. A homozygous null mutation in *ABP1* conferred embryo lethality. The embryos developed normally until the early globular stage, but did not progress to bilaterally symmetrical embryos because of failure of cells to elongate. Cell division was also aberrant. Furthermore, antisense suppression of ABP1 in tobacco cells eliminated auxin-induced cell elongation and caused a reduction in cell division. These data are the most convincing proof yet of a role for ABP1 in auxin-mediated cell enlargement and possibly cell division, though whether they signify that it is an auxin receptor or some essential early component of auxin signaling is unclear.

Other supporting data include detection by immunostaining of small amounts of *Zm*-ABP1 on the external face of the plasma membrane of maize protoplasts. Because the deduced amino acid sequences for ABP1 show no sequences typical of an integral membrane protein (see Fig. 22-3), it is further suggested that ABP1 is anchored to a separate "docking", transmembrane protein in the plasma membrane.

2.1.2.3. Arguments against ABP1 as an Auxin Receptor

Despite the abundant circumstantial evidence that ABP1 is an auxin receptor, many scientists remain skeptical that it is an auxin receptor.

i. ABP1 and auxin-induced gene induction. If ABP1 is a receptor for auxin, there should be some direct connection between auxin binding to ABP1 and induction of some of the early auxin responsive genes, such as the *Aux/IAA* class of genes (see Section 3). However, up to now this has not been demonstrated.

ii. Binding of ABP1 to auxins shows much variation. *In vitro* binding experiments show that *Zm*-ABP1 binds 1-NAA with high affinity ($K_D \approx 5 \times 10^{-8} M$),

but its affinity for IAA, the principal naturally occurring auxin, is 10- to 20-fold less; for some of the other synthetic auxins, such as 2,4-D, it is much lower.

iii. Abundance of ABP1 in different plants. It is surprising that a protein believed to be an auxin receptor should show such disparate occurrence in different plants. It is abundant in maize and is present in all parts of the plant except root tips, but present in very low amounts in other plants, including all dicots examined to date. However, auxins are active in both monocots and dicots, and one would expect that an auxin receptor should show similar distribution in most plants.

iv. No adequate explanation is available as to how an ER luminal protein reaches the cell surface. There is no doubt that the great bulk of *Zm*-ABP1, more than 98%, is located in the endoplasmic reticulum. This has been confirmed by immunological, including immunofluorescence, studies. It has been suggested that auxin binding to ABP1 causes a conformational change, such that the KDEL sequence is hidden, and the protein escapes the salvage machinery in the *trans*-Golgi apparatus, but there is little direct evidence for such binding. Optimal binding of auxins to the site I protein (also purified *Zm*-ABP1) occurs at ~pH 5.5 (see Fig. 22-2), not at the pH of the ER (~pH 7.0). It may be that small amounts of the protein do escape from the ER and *trans*-Golgi network and end up at the cell surface, but a satisfactory explanation is lacking.

2.1.3. Other Membrane-Bound ABPs

Several other auxin-binding proteins, in addition to ABP1, have been tagged with photoaffinity-labeled IAA. As explained in Chapter 13, some of these proteins are involved in auxin uptake or efflux. Others include a protein, which is a β -glucosidase capable of hydrolyzing cytokinin-glucose conjugates, not auxin conjugates. Still others include a superoxide dismutase, a β -1,3-glucanase, and both soluble and membrane-bound glutathione S-transferases. The likelihood of any of these proteins being involved in auxin signal perception or transduction is small.

The isolation of these proteins after photolabeling with tritiated azido IAA ($[^3H]N_3IAA$) and precautions that binding was specific highlight the difficulties in the isolation of receptor proteins. It also means that two or more methodologies are required for a reliable identification of any protein as a receptor protein.

2.2. An Intracellular Site for Auxin Perception

It has been suggested that while electrical responses of isolated protoplasts or guard cells may well involve

an extracellular site for auxin perception, the elongation response as shown by stem or coleoptile segments involves perception of auxin at an intracellular site. As mentioned in Chapter 13, phytoalexins, such as naphthylphthalamic acid (NPA), block IAA efflux from cells and thus raise the intracellular IAA concentration (see Fig. 13-14). Some authors have used this technique to show that cell growth in stem segments is correlated more closely with the intracellular concentration of IAA than with extracellular IAA. Other arguments for an intracellular site derive from mutants that are insensitive to auxin. The *aux1* mutants of *Arabidopsis* are defective in auxin uptake, and several auxin-induced responses, such as inhibition of root growth or induction of lateral roots, are slowed down (see Chapter 13). Such slowing down of auxin responses is an argument for an intracellular site for auxin perception.

2.3. Soluble Auxin-Binding Proteins

Several soluble proteins, i.e., proteins in $100,000 \times g$ supernatant from tissue extracts, which bind 3H -IAA with high specificity and affinity, were characterized in dividing cells of tobacco pith cultures. Other soluble IAA-binding proteins have been reported in tissue extracts from elongating pea epicotyls, rice shoots, and 2,4-D-binding proteins from mung bean hypocotyls. Most of these proteins were not purified, but a 57-kDa protein from rice has been purified and shown to bind IAA with high affinity ($1.9 \times 10^{-8}M$). Moreover, the protein was shown to induce H^+ -ATPase activity in microsomal fractions from rice, an activity that was significantly increased with the addition of IAA. As expected, IAA alone showed no induction of ATPase activity. In view of the controversy surrounding ABP1 as an auxin receptor, perhaps the soluble proteins should be investigated again.

2.4. Summary of Auxin Perception

In summary, there is abundant correlative evidence that ABP1, specifically *Zm*-ABP1, is an auxin receptor. Antibodies against *Zm*-ABP1 inhibit the auxin-induced hyperpolarization response in protoplasts. Use of impermeant auxins supports the idea that this inhibition is due to the binding of antibodies to auxin receptors that are located on the external face of the plasma membrane. It seems likely that *Zm*-ABP1 is that receptor because (i) antibodies against a conserved region of ABP1 bring about a hyperpolarization response, similar to that induced by auxins, (ii) a peptide from ABP1 binds photolytically reactive azido IAA, and (iii) a peptide based on the C terminus of *Zm*-ABP1 modulates the K^+ current in intact guard

cells of *Vicia faba* in a manner reminiscent of auxins. In a more direct demonstration of the role of ABP1 in auxin-induced cell enlargement, a homozygous null mutant of *ABP1* in *Arabidopsis* confers embryo lethality because cells at early globular stage fail to elongate. Also, antisense suppression of ABP1 in transgenic tobacco similarly causes a lack of cell elongation and reduces cell division. As shown immunologically, it is present in all parts of maize plants except root tips. Finally, in accord with the postulated surface receptor, small amounts of *Zm*-ABP1 have been immunolocalized on the cell surface of maize protoplasts.

One of the major weaknesses in the postulate that ABP1 is an auxin receptor is that ABP1 has not been shown to be involved in the other very fast response to auxins, i.e., induction of certain genes. Other objections relate to the binding affinity of the ABP1 protein to natural and synthetic auxins, as well as its relative abundance in major groups of plants. Finally, no satisfactory explanation is yet available as to how a protein that is resident in the ER reaches the surface of the plasma membrane.

It remains possible that intracellular sites for auxin perception exist. It is also possible, given the multiple roles of natural auxins in plant growth and development, that multiple pathways for auxin perception and signaling are present. In the next section on auxin-induced genes we will see that there are combinations of regulatory elements that make such multiple signaling possible.

3. MOLECULAR GENETIC APPROACH

While the search for an auxin receptor continues, molecular and genetic techniques have provided some important components in auxin signaling and confirmed the role of auxin in regulating developmental and tropic responses and in patterning. We begin by introducing some auxin response mutants and the screens used to select them and then proceed to a consideration of the genes that are expressed early in the auxin response and the *cis* sequences and transcription factors involved in auxin signaling. This is followed by a consideration of genes and encoded proteins identified by response mutants and our current understanding of auxin signaling.

3.1. Auxin Response Mutants and Screens

Mutants with an altered response to auxin are known from tomato, tobacco (*Nicotiana plumbaginifo-*

lia), and *Arabidopsis*. Those in *Arabidopsis* are best characterized and are covered here.

As explained in Chapters 14 and 15, auxins inhibit the elongation growth of roots at concentrations that stimulate lateral root initiation and growth of stems. At an IAA concentration of 50 μM or a 2,4-D concentration of 5 μM , the seeds germinate, cotyledons expand, but root elongation is severely inhibited. Accordingly, one of the screens to obtain auxin response mutants is to grow mutagenized seeds in a medium supplemented with high concentrations of auxin (e.g., 5 μM 2,4-D or 50 μM IAA) and select seedlings whose root growth is not inhibited or inhibited only slightly. Some mutants selected in this manner are defective in IAA uptake (*aux1*); they are covered in Chapter 13, along with auxin efflux mutants. Other mutants, auxin-resistant (*axr*) mutants, are thought to represent lesions in auxin perception or signal transduction. Some of these *axr* mutants have turned out to be mutations in genes that are expressed very rapidly after auxin supply, the primary auxin response genes, whereas others encode proteins that seem to play a role in the targeted degradation of other proteins. Both are part of auxin signaling and are covered in this chapter. In Chapter 13, mention was also made of *tir* (for transport inhibitor response) mutants that are tolerant to relatively high doses of inhibitors of polar auxin transport (e.g., NPA). *tir* Mutants belong to several genetic loci. One locus, *TIR3*, plays a role in auxin transport, but another, *TIR1*, is important in signaling as part of a complex involved in protein degradation.

As stated earlier, stems and petioles have different thresholds for auxin response than root growth. To get a more complete picture, therefore, it is important to have mutants that are impaired in responses by an aboveground part. It is well known that stems placed horizontally grow upward (negative gravitropism). This change in orientation results from greater growth on the lower, than on the upper, side of the stem and is thought to result from a greater concentration of auxin on the lower side (see Chapter 27). Unilateral application of indoleacetic acid (IAA) in a lanolin base to hypocotyls of etiolated *Arabidopsis* seedlings induces a growth curvature similar to that in a gravitropic response. *massugu1* (*msg1*) Mutants were selected because they do not undergo hypocotyl growth curvature at any of the IAA concentrations tested (to 1000 μM), yet the roots respond normally. The mutants are also normal in their responses to other hormones, such as ethylene and ABA.

The mutations just described and some related ones and their wild-type genes are covered in

Section 4 following a consideration of auxin-regulated genes.

4. AUXIN-INDUCED GENES

Since the first cloning of auxin-induced genes by Ainley *et al.* (1988), the number of genes reported to be induced by auxin has increased phenomenally. However, not all of these genes are regulated at the transcriptional level, and only some can be considered as being regulated directly by auxins.

Transcriptionally induced genes fall into two broad groups. One group whose transcripts accumulate to detectable amounts within minutes of auxin treatment, usually less than 60 min, are referred to as "early induced" genes. Others are induced later and are referred to as late-induced genes. Early induced genes are particularly important because their induction is closer to the primary site of auxin action. The later-acting genes are probably triggered by events unleashed by early induced genes; they are considered in this chapter only in passing.

4.1. Early Induced Genes

Among early induced genes, some are also classed as primary auxin response genes, i.e., their induction is not dependent on new synthesis of any other protein (see Appendix 1). Primary response genes are widely diverse and fall into several classes depending on the nature of proteins encoded. To some extent, this is also a reflection of the auxin used, whether it is indoleacetic acid or 2,4-D, and whether low or high concentrations of 2,4-D are used. At low concentrations, 2,4-D brings about some of the same responses as IAA, but at high concentrations it is toxic and acts as a herbicide to most broad-leaved plants (dicots) and elicits defense responses.

Table 22-2 lists some of the early auxin-induced genes. They come from elongating systems of pea stems, mungbean, soybean hypocotyls, and *Arabidopsis*, and additional members have been isolated from *Arabidopsis* and tobacco using homology cloning. Three classes are shown: *Aux/IAA*, *SAUR*, and *GH3*. Members of these gene families have little in common with each other in terms of structure and function, but they are placed together in Table 22-2 because they are rapidly induced by auxins and their expression is not inhibited by protein synthesis inhibitors. These genes are not only expressed after auxin application but, in plants overproducing IAA

TABLE 22-2 Representatives of Major Classes of Early Auxin-Induced Genes, Inducing Auxin, and Plant Species^a

Gene	Inducing hormone	Plant species	Response time ^b (min)	Sensitivity to CHX ^c	Other inducers ^d
Aux/IAA gene family					
<i>Aux22</i>	2,4-D (23 μ M)	<i>Glycine max</i>	15	n.d. ^e	n.d.
<i>Aux 28</i>			30		n.d.
<i>At-Aux2-11 (IAA4)</i>	IAA	<i>Arabidopsis thaliana</i>	30	n.d.	n.d.
<i>At-Aux2-27 (IAA5)</i>			90	n.d.	n.d.
<i>ARG3</i>	IAA	<i>Vigna radiata</i>	20	n.d.	CHX
<i>ARG4 (Vr-Aux22B)</i>	(20 μ M)		20	n.d.	CHX
<i>GH1</i>	2,4-D (50 μ M)	<i>G. max</i>	15	Insensitive	—
<i>Ps-IAA4/5</i>	IAA (20 μ M)	<i>Pisum sativum</i>	5	Insensitive	CHX
<i>Ps-IAA6</i>			8		CHX
<i>Nt-AUX8, Nt-AUX16</i>	Cloning by homology	<i>Nicotiana tabacum</i>	n.d.	n.d.	n.d.
<i>IAA1-IAA6</i>	Cloning by homology	<i>A. thaliana</i>	5–25	Insensitive	CHX
<i>IAA7, IAA8</i>			60–120	Sensitive	—
<i>IAA9-IAA14</i>			15–60	Insensitive	CHX
SAUR gene family					
<i>SAURs</i>	2,4-D	<i>G. max</i>	3–5	Insensitive	CHX
<i>ARG7</i>	IAA (20 μ M)	<i>V. radiata</i>	5	n.d.	CHX
<i>SAUR-AC1</i>	Cloning by homology	<i>A. thaliana</i>	n.d.	Insensitive	CHX
GH3 gene family					
<i>GH3</i>	2,4-D	<i>G. max</i>	5–15	Insensitive	—

^aModified from Abel and Theologis (1996). Some genes have been redesignated as shown in parentheses.

^bResponse time as detected by nuclear run on transcription or by changes of steady-state mRNA levels.

^cGene expression is inhibited (sensitive) or not inhibited (insensitive) by prior treatment with cycloheximide (CHX).

^dA dash indicates that none of the agents tested other than active auxins are able to elicit a response.

^eNot determined.

(e.g., tobacco plants transformed with bacterial *iaaM* and *iaaH* genes), they can be expressed constitutively.

4.1.1. Aux/IAA Genes

Members of the *Aux/IAA* gene family have been identified in several plants (e.g., soybean, *Arabidopsis*, mung bean, pea, and tobacco). Detailed studies on two genes from pea, *Ps-IAA4/5* and *Ps-IAA6*, indicate that they encode polypeptides with a very short half-life ($t_{1/2}$ of ~6 to 8 min). Moreover, their expression patterns correspond with the presumed sites of auxin action in plants. For instance, in tobacco seedlings transformed with constructs consisting of the promoter region of *Ps-IAA4/5* or *Ps-IAA6* and a reporter gene, the reporter gene was expressed primarily in zones of cell division (e.g., root tips and sites of initiation of lateral root primordia), in the zone of elongation (i.e., the subapical region) of etiolated hypocotyls, and in vascular tissues (Fig. 22-6). These are precisely the regions where IAA and other auxins are thought to be active (see Chapters 14 and 15).

Aux/IAA genes are primary auxin response genes because their expression is not inhibited by cycloheximide (CHX). In addition, they are also induced by CHX (see Table 22-2), which suggests that the transcription machinery for these genes, i.e., the basal initiation complex and gene-specific transcription factors are already in place, but that they are negatively regulated by a repressor protein. In the presence of auxin, the repressor is deactivated such that transcription can begin. The nature of the repressor or its mode of action is unknown, but there is evidence that auxin signaling involves protein degradation (see Section 6).

Arabidopsis has more than 20 *IAA* genes. The kinetics of expression and the patterns of tissue-specific expression vary among different genes. Thus, it seems likely that different members of the family have different roles in auxin response.

Aux/IAA genes encode small, hydrophilic polypeptides with molecular masses of 19 to 36 kDa. As judged from immunological studies, the proteins are present in small amounts and show a rapid turnover.

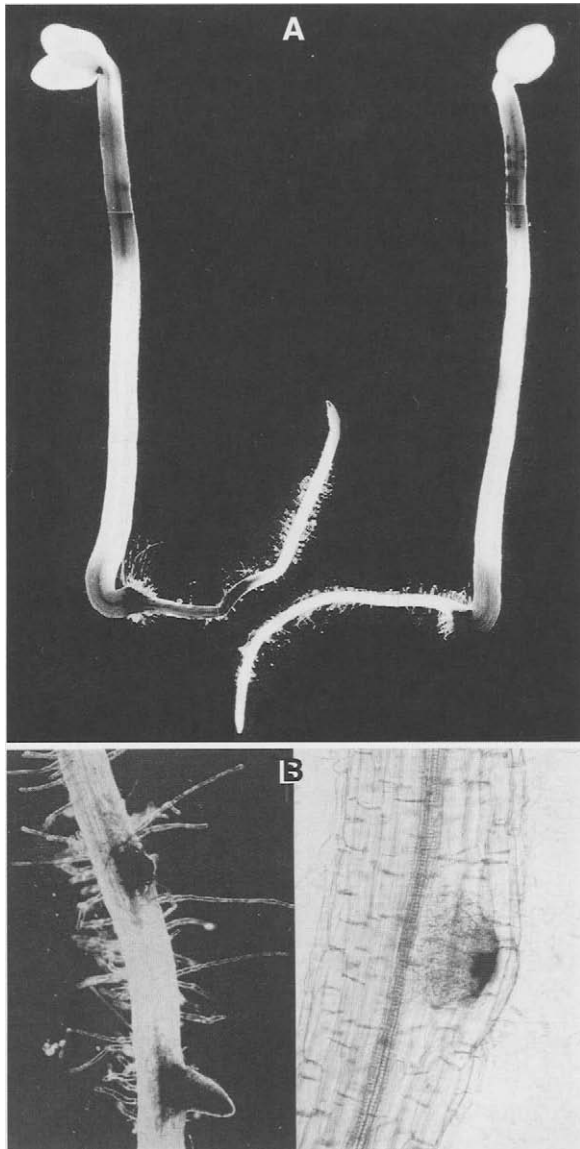


FIGURE 22-6 Expression of *Ps-IAA4/5* and *Ps-IAA6* genes during hypocotyl elongation and lateral root initiation. Fusion constructs consisting of the promoter region of the *Ps-IAA4/5* or *Ps-IAA6* gene from pea (*Pisum sativum*) and the coding region of the *GUS* gene were used to transform tobacco plants using *Agrobacterium tumefaciens*. Expression of *GUS* (blue color of *GUS* reaction appears as a dark patch) was monitored during the normal growth of seedlings. (A) Hypocotyl growth in dark-grown seedlings, transformant *Ps-IAA4/5*. (B) Lateral root initiation, transformant *Ps-IAA6*. Reprinted with permission from Wong *et al.* (1996), © Blackwell Science Ltd.

Sequence comparisons of these proteins from different plants show four highly conserved domains, domains I–IV, and two motifs, nuclear localization signals (NLS) and an amphipathic $\beta\alpha\alpha$ fold, which forms part of domain III (Fig. 22-7). The $\beta\alpha\alpha$ motif also occurs in certain prokaryotic repressors of gene transcription where it is involved in DNA binding as well as dimerization with similar units.

The presence of nuclear localization signals and indications of a putative DNA-binding domain in Aux/IAA proteins are suggestive of a role as transcription factors, but they have not been shown to bind DNA. Instead, using both yeast two hybrid screens and *in vitro* experiments, it has been shown that these proteins are able to form homo- and heterodimers with each other and that the C terminus with domains III and IV is involved in dimerization.

4.1.2. Aux/IAA Proteins Play Important Roles in Plant Development

It was mentioned earlier that some auxin response mutants have turned out to be mutations in *Aux/IAA* genes. At least six such loci are known, including *SHY2/IAA3*, *AXR2/IAA7*, and *AXR3/IAA17*. Partially dominant mutations in these genes cause pleiotropic but distinct phenotypes affecting leaf size and shape, branching patterns in inflorescence axes, root growth, production of lateral roots and/or root hairs, gravitropic response, and photomorphogenesis in dark. For instance, *axr2* mutants are green and robust, the leaves are smaller, rounder, with shortened petioles, and the inflorescence axes are dwarfed in comparison to the wild type (Fig. 22-8). The mutant also produces far more lateral roots than the wild type at 10^{-5} M IAA. The *axr3* mutants produce curled epinastic leaves, an inflorescence axis, which shows a reduced stature but strong apical dominance (see Fig. 22-8), and far more adventitious roots, which remain short and agravitropic compared to the wild type. The mutant also shows ectopic expression of *SAUR* genes (for *SAUR* genes, see Section 4.1.3). Notably, the mutant can be partially restored to the wild type by the treatment of seedlings with cytokinin.

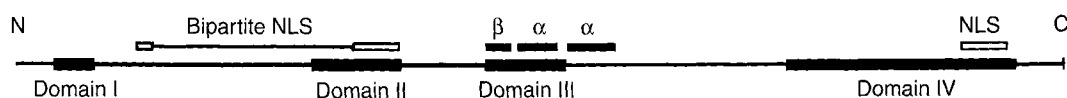


FIGURE 22-7 Structural map of a representative Aux/IAA polypeptide. Four conserved domains, designated I–IV, are shown. Domain III includes part of the $\beta\alpha\alpha$ motif. Nuclear localization signals (NLS) are shown as open boxes; one NLS is bipartite. Domains III and IV are involved in dimerization. Adapted with permission from Rouse *et al.* (1998), © American Association for the Advancement of Science.

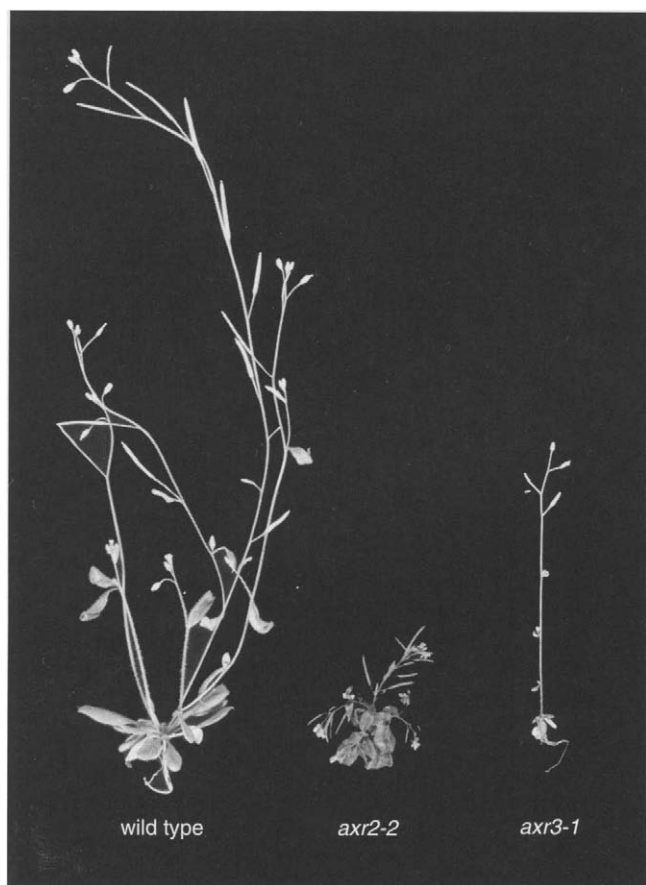


FIGURE 22-8 Phenotypes of wild type and *auxr2-2* and *auxr3-1* mutants. From Wilson *et al.* (1990) and Leyser *et al.* (1996).

These mutant phenotypes provide genetic evidence that the encoded proteins of several *Aux/IAA* genes are involved in auxin responses. Moreover, they seem to regulate distinct aspects of auxin responses. How they do so is unknown, but there is increasing evidence that they serve as transcriptional regulators, either directly or by interacting with other transcription factors. There is also evidence that some of them may negatively regulate an auxin response and thus may be subject to proteolytic degradation. These topics are discussed further in Sections 5 and 6.

4.1.3. *SAUR* Genes

SAUR (Small auxin-upregulated) genes were first identified in soybean after 2,4-D treatment, but their homologues are now known from other plants, including mung bean, pea, and *Arabidopsis*. Their induction is one of the most rapid after applying auxin to auxin-depleted tissue, and their expression may reach half-saturation within 10 min. As shown in Table 22-2,

SAUR gene expression is not inhibited by cycloheximide. *SAUR* genes are a family of small genes, about 550 bp with no introns, which in some species occur in clusters and which encode highly similar polypeptides of 9 to 10 kDa. The function of *SAUR* polypeptides remains unknown, and sequence comparison in data bases, either at the gene or polypeptide level, has yielded no clues. Tissue printing or the *in situ* hybridization technique shows that their mRNAs are induced preferentially in epidermal and cortical cells and, further, that during a tropic response (gravi- or phototropism), their mRNAs accumulate selectively in cells toward the side that is showing more growth. Fusion constructs of 5' upstream sequences of these genes with reporter genes, e.g., *GUS*, have confirmed these results.

4.1.4. *GH3* Gene Family

GH3 genes comprise a small family in soybean, and related genes are reported in *Arabidopsis*. They are induced rapidly by auxin, and their expression, like that of *Aux/IAA* and *SAUR* genes, is insensitive to cycloheximide (see Table 22-2). However, in contrast to *Aux/IAA* and *SAUR* genes, their expression is not induced by cycloheximide. They also encode much larger proteins (~70 kDa), which are produced in larger amounts and are much more stable. Their expression patterns are also different. In tobacco seedlings transformed with *GH3* promoter::*GUS* constructs, without NAA treatment, they were expressed only in ovules, developing seeds, and in vascular tissues of flower peduncle. On induction by NAA, they showed strong expression in hypocotyls, proximal parts of cotyledons and shoot apex, petioles, vascular tissues, and developing seeds. They show no sequence similarity to any sequences in data banks, and their functions remain unknown.

4.2. Other Genes

In addition to genes that are induced early and are primary auxin response genes, many other genes are also induced by auxins, some as early as 10 min after application of auxin, others later (12–24 h). Among these, some encode proteins that are related to known auxin functions, such as cell wall loosening, ethylene biosynthesis, and induction of cell cycle regulatory proteins; others encode proteins of doubtful or unknown relevance to auxin function. Still other genes are downregulated. A brief account of these genes is provided in Box 22-1, but they are not the focus of this chapter.

BOX 22-1 OTHER AUXIN-REGULATED GENES

A. Genes Encoding Proteins Related to Known Auxin Functions

i. Proteins associated with cell walls. Xyloglucan endotransglycosylases (XETs, also called endoxylan transferases or EXTs) and β 1,4-glucanases are involved in wall loosening during cell growth (see Chapter 15). IAA ($1.0\ \mu\text{M}$) treatment of *Arabidopsis* seedlings has been shown to induce two genes encoding XETs (*TCH4* and *EXT*) within 10–30 min of application. Likewise, a gene from pea hypocotyls (*EGL1*), encoding a β 1,4-glucanase, has been shown to be induced by 2,4-D ($5.0\ \mu\text{M}$).

Hydroxyproline- and proline-rich proteins (HRGPs and PRPs) are important wall constituents and are believed to stabilize the cell wall architecture in mature cells (see Chapter 2). Genes encoding the protein moieties of HRGPs and PRPs have been isolated from roots of carrot, soybean, and tobacco after auxin treatment ($10\ \mu\text{M}$ IAA, NAA, 2,4-D).

ii. Calcium-binding proteins. Calcium plays an important role in mediating several physiological processes, including signal transduction, in plants. It does so by activating certain proteins, which either themselves act as protein kinases (e.g., calcium-dependent protein kinases, CDPKs) or which, in turn, modulate the activities of protein kinases or phosphatases (see Chapter 25). The latter group includes such calcium-binding proteins as calmodulin (Cam). Some genes encoding calmodulin or CDPKs have been shown to be induced by IAA.

iii. Genes associated with cell cycle. As explained in Chapter 15, several hormones, IAA, GAs, and CKs, are known to induce cell division by regulating the expression of cyclins and/or CDC2 kinases. Several CDC2 genes have been reported to be induced by auxins.

iv. Ethylene biosynthesis. Auxins are known inducers of ethylene biosynthesis, specifically by regulating the expression of genes coding for ACC synthase (see Chapter 11). A gene encoding ACC synthase in *Arabidopsis* seedlings has been cloned and shown to be a primary auxin response gene. It is induced within 20–25 min of auxin application, and it was also induced by cycloheximide. Other hormones, ABA, GA, a cytokinin (benzyladenine), and ethylene, are ineffective.

B. Genes of Doubtful or Unknown Relevance to Auxin Action

i. Glutathione S-transferases (GSTs) and GST-like proteins. A series of genes have been isolated from soybean hypocotyls (*GH2/4*), from tobacco (*Nicotiana tabacum*) cell culture (*cnt103*, *cnt107*), and from tobacco leaf mesophyll protoplasts, (protoplast auxin responsive or *PAR*), which are induced by auxins, such as 2,4-D and NAA. These genes were initially isolated in screens for auxin-induced cell division, but most likely have no such role. Instead, they have been shown to encode GSTs or GST-like proteins. GSTs are a class of enzymes involved in plant defense reactions, including detoxification of xenobiotics and herbicides. Hence, it is not surprising that these genes are also induced by a variety of other signals, such as wounding, pathogen attack, and ethylene.

ii. Miscellaneous genes. Genes encoding oxidative enzymes, such as ascorbate oxidase and oxalate oxidase; an enzyme involved in the synthesis of unsaturated fatty acids (a fatty acid desaturase, *FAD*); and many others, have been linked to auxin application, but their role in auxin-mediated functions remains obscure.

C. Auxin-Induced Downregulation of Genes

In addition to upregulated genes, many other genes have been shown to be downregulated by auxin application. These include genes encoding enzymes in secondary metabolism, such as tryptophan decarboxylase (TDC), phenylalanine ammonia lyase (PAL), and chalcone synthase (CHS), and pathogenesis-related proteins, such as endo- β -1,3-glucanases and chitinases, as well as a family of auxin-downregulated (ADR) genes of unknown function in soybean. How these genes are downregulated is not known.

4.3. Regulation of Early Auxin-Induced Genes

The cloning and sequencing of members of the *Aux/IAA*, *SAUR*, and *GH3* gene families have led to the identification of conserved *cis* sequences in their promoter regions. As mentioned earlier, these gene families have little structural similarities, but they show similar patterns of regulation. In the following, the *cis* elements in *Ps-IAA4/5*, an *Aux/IAA* gene, and *GH3* gene are described, followed by isolation of auxin-specific transcription factors.

4.3.1. *cis* Elements and Transcription Factors

Analysis of promoter segments of the *Ps-IAA4/5* gene has identified a consensus sequence, 5'-T/GGTCCCAT-3', which is present in the promoter regions of many primary auxin responsive genes and which is regarded as a core auxin response element (AuxRE). This AuxRE probably functions in association with other *cis* elements for full auxin responsive expression.

Similar studies with the *GH3* promoter have yielded two response elements: D1 and D4. Each contains a 5'-TGTCTC-3' element, which, although necessary, is not sufficient by itself to confer auxin responsive expression to a minimal promoter. It requires an adjacent or an overlapping coupling element to form a functional unit, an auxin response complex (AuxRC) (Fig. 22-9).

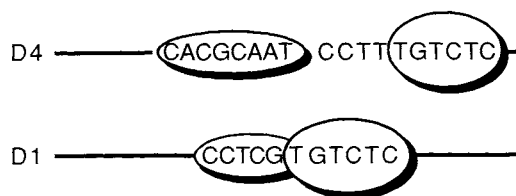


FIGURE 22-9 Auxin response elements in the promoter of the *GH3* gene. Two elements, D1 and D4, each contain a TGTCTC sequence. Both have a coupling element that is separated from the TGTCTC element in D4, but overlaps that element in D1. Modified from Ulmasov *et al.* (1995).

The TGTCTC sequence is palindromic, and inverted repeats of palindromic sequences often bind transcription factors. Hence, a synthetic palindromic TGTCTC repeating sequence was used as bait in a yeast one-hybrid system to isolate an auxin-specific transcription factor from *Arabidopsis*. It was called auxin response factor 1 (ARF1). Since the first report in 1997, at least 10 ARFs have been isolated or identified in *Arabidopsis*. They all bind to the TGTCTC element and are specific to the auxin response. They vary in length, but generally are much larger proteins than the Aux/IAA proteins. Their deduced amino acid sequences show an N-terminal DNA-binding domain, followed by a variable region, and, with few exceptions (e.g., ARF3), a carboxy terminus, which shows the conserved domains III and IV of Aux/IAA-type proteins (Fig. 22-10).

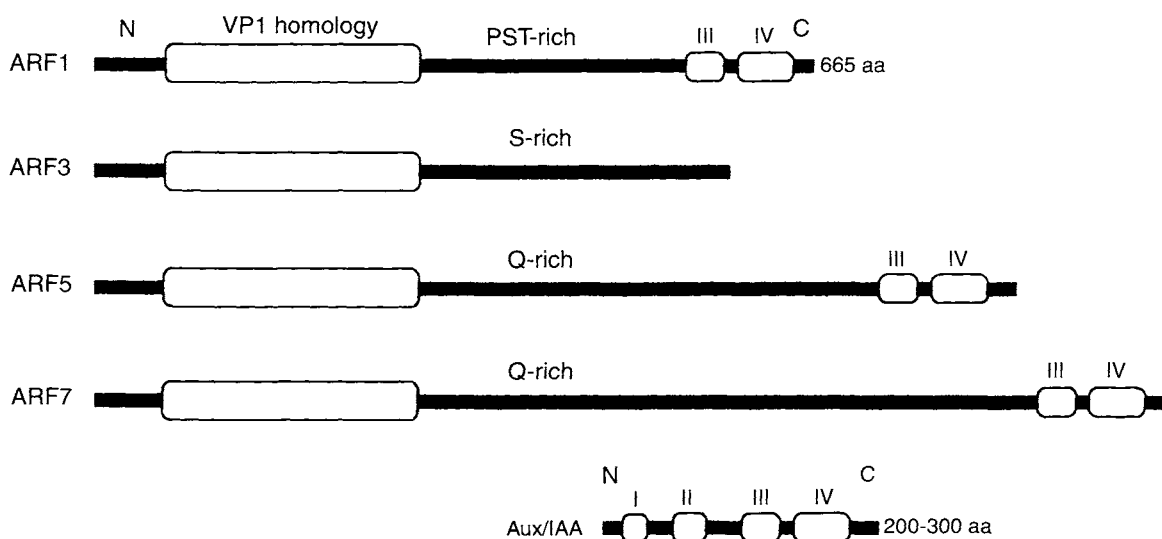


FIGURE 22-10 Schematic diagrams of selected ARF1 and related proteins. The DNA-binding domain near the N terminus is similar to the DNA-binding domain of maize VP1, a transcription factor involved in ABA signaling (see Chapter 23). It is followed by a variable region rich in specific amino acids (designated by single letter symbols). Boxes III and IV are homologous to domains III and IV in Aux/IAA proteins. Note that ARF3 lacks domains III and IV. Modified from Ulmasov *et al.* (1997).

4.3.2. Auxin Response Factors Are Important Determinants of Developmental Phenomena in Plants

At least three of the ARFs identified to date in *Arabidopsis* are identical to the wild-type genes of mutants defective in responses thought to be auxin mediated. *ettin* (*ett*) Mutations have several defects in *Arabidopsis* flower development, including organ number, and apical-basal patterning in the gynoecium. The *ETTIN* gene is identical to *ARF3*, and following the rules of nomenclature, the gene is now referred to as *ETTIN/ARF3*. The *monopteros* (*mp*) mutant shows a lack of axialization in embryo development and defective vasculature in the adult plant (see Chapter 14). The *MONOPTEROS* gene is identical to *ARF5* and is now known as *MP/ARF5*. The *msg1* mutant is allelic to *nph4* (for nonphototropic hypocotyl, see Chapter 27), and the wild-type gene *MSG1/NPH4* is identical to *ARF7*. It is now known as *MSG1/NPH4/ARF7*.

These examples serve to emphasize and confirm, first, that auxin is involved in several aspects of plant developmental and tropic responses and in embryo and vascular patterning. Second, that different ARFs regulate specific phenotypes and that mutations in these factors have far-reaching developmental and physiological consequences. The roles of other ARFs in *Arabidopsis* are still unknown and, of course, we know little about auxin-specific transcription factors in other plants.

4.3.3. Combinatorial Control of Auxin Signaling

As mentioned in Appendix 1, homo- or heterodimerization of transcription factors, together with combinations of *cis* elements in a modular fashion, provides the needed diversity and specificity for hormone- and tissue-specific gene transcription. Because Aux/IAA proteins and auxin response factors (ARFs) both carry the carboxy terminus domains III and IV, they can form homo- or heterodimers within the same protein type or between the two types (Fig. 22-11). Given the relatively large numbers of the two types of proteins (in *Arabidopsis*), the number of possible combinations that can be used for tissue-/organ- or development-specific auxin signaling is enormous. Aux/IAA proteins have a putative DNA-binding motif and could bind DNA. They have not been shown to bind DNA on their own; hence, it is suggested that they bind to ARFs, which, in turn, bind to DNA. However, both possibilities are open and are shown in Fig. 22-11.

Whether such combinations occur *in planta* in response to auxin availability, and what the downstream targets for such regulation might be are topics that are being actively pursued. The technique of targeted genetics (see Appendix 1) allows retrieval of mutants that are specific to a particular gene and, thus, can be useful in providing information on proteins that interact with the product of that gene. In one such effort, the *PsIAA4/5::GUS* construct was used to stably transform *Arabidopsis* plants. One of the transformed lines that showed a good auxin response was mutagenized, and the mutants were screened for an altered response to exogenous auxin. Two mutants, both single gene recessives, called *age* (for altered gene expression), have been obtained. Cloning of the wild-type *AGE* gene may reveal upstream elements that affect the activity of the *Ps-IAA4/5* promoter.

5. AUXIN SIGNALING INVOLVES TARGETED PROTEIN DEGRADATION

The *axr* mutations isolated so far represent lesions at several loci. Two of these, *axr2* and *axr3*, are dominant or semidominant mutations. The wild-type genes, *AXR2* and *AXR3*, as mentioned earlier, are identical to *Arabidopsis* *IAA7* and *IAA17* genes, respectively. Thus, they encode Aux/IAA-type proteins. Two other mutant loci, *axr1* and *axr4*, have yielded recessive mutations. Among the two, *axr4* affects mainly the root system, whereas *axr1* is pleiotropic in its effects. Less is known about the *AXR4* than about the *AXR1* locus; hence, the following discussion pertains mostly to *AXR1* and loci associated with it. Mutants at a new locus, *AXR6*, were identified recently; they show defects in early embryogenesis (see Chapter 14).

5.1. AXR1

Mutant alleles of *AXR1* with mild (*axr1-3*) or severe effects (e.g., *axr1-12*) on the phenotype are known. Mutants in the homozygous state have small, curling leaves with shortened petioles; long, unbranched roots, which show impaired gravitropism; and inflorescence axes that are highly branched and reduced in height, with an overall effect similar to loss in apical dominance (Fig. 22-12). Some data indicate that the

mutant is not affected in initiation or timing of initiation of lateral bud meristems, but in their growth. Mutant seedlings grown in the dark show an inhibition of hypocotyl elongation.

Bioassays confirm reduced auxin sensitivity in all responses measured. As expected from the screen, the *axr1* mutant requires a higher concentration of IAA to inhibit root elongation growth than the wild type (Fig. 22-13A). At the same concentration of IAA, fewer lateral roots are initiated in the mutant than in the wild type (see Fig. 22-13B). The expression of primary response genes, such as *Aux/IAA* or *SAUR* genes in the mutant, requires a much higher concentration of auxin than in the wild type. As is typical for insensitive

response mutants, the endogenous auxin levels are somewhat higher than in the wild type. All of these features suggest that the *axr1* mutation results in a diminished sensitivity of plant tissues to endogenous or applied auxin. Since it affects multiple responses, the *AXR1* locus is thought to act at an early step in auxin signaling.

The *AXR1* gene encodes a protein (molecular mass about 60 kDa) that shows similarity to the N-terminal half of a ubiquitin-activating enzyme (E1) from yeast. Ubiquitins are small proteins that occur universally in organisms. They bind to proteins and target them for degradation by the 26S proteasome (see Box 22-2).

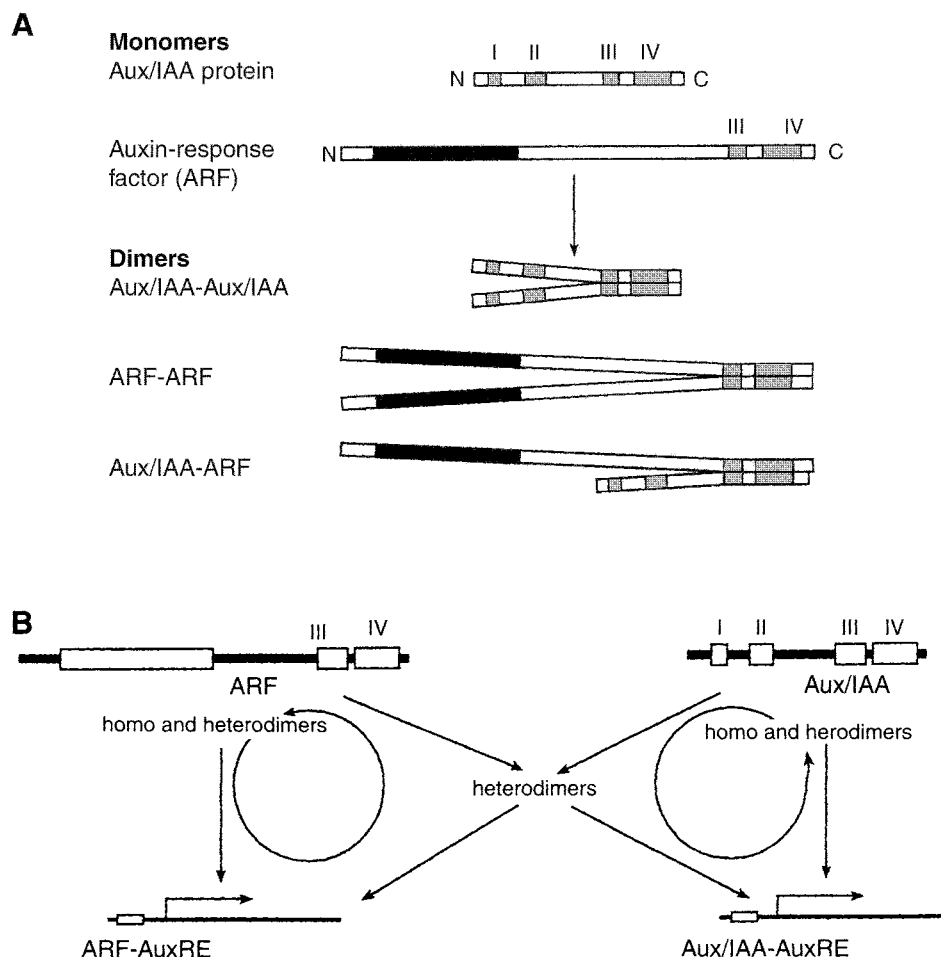


FIGURE 22-11 Schematic drawings showing dimerization of ARF and Aux/IAA proteins and their potential interactions in regulating gene transcription. (A) Homo- and heterodimers of ARF and Aux/IAA. Conserved domains are shaded. From Guilfoyle (1998). (B) Possible modes of interaction between members of Aux/IAA and ARF families of proteins in gene transcription. Modified from Leyser and Berleth (1999) with permission from Elsevier Science.



FIGURE 22-12 Phenotypes of wild type and two *axr1* mutants, one with a severe allele and the other with a weak allele. Mature plants of: (A) wild type, (B) homozygous *axr1-12*, and (C) homozygous *axr1-3*. Bar: 3 cm. From Lincoln *et al.* (1990).

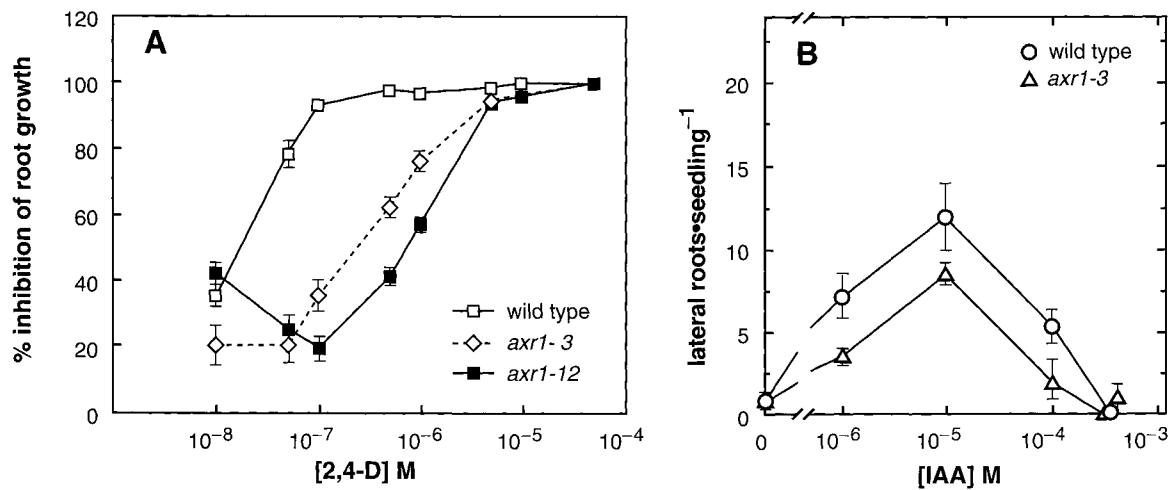


FIGURE 22-13 Sensitivity of auxin responses in *Arabidopsis* roots. (A) Inhibition of root elongation by 2,4-D in wild type and in homozygous *axr1-3* and *axr1-12* mutants. Note that the *axr1-12* mutant requires almost a 100 times greater auxin concentration than the wild type for 50 or 100% inhibition of root growth. From Lincoln *et al.* (1990). (B) IAA dose-response curves for lateral root production in wild-type and *axr1-3* seedlings. Seeds were germinated and grown in darkness for 3 days and then transferred to media containing IAA. Data are the mean \pm SE for the number of roots per seedling 5 days after transfer. Data shown are from one of three replicate experiments with 10 seedlings per treatment. From Knee and Hangarter (1996).

BOX 22-2 UBIQUITINIZATION AND DEGRADATION OF PROTEINS

UBIQUITIN IS A 76 amino acid protein that targets cellular proteins for degradation by the 26S proteasome machinery in nearly all organisms. Ubiquitin-conjugation to target proteins occurs in three steps (Fig. 21-14). In the first step, ubiquitin is activated and linked by a thioester bond between its C terminus and a cysteine residue within an enzyme, E1, the ubiquitin-activating enzyme. Activation requires ATP hydrolysis into AMP and PP_i and use of hydrolysis energy to link up the thiol ester bond. In the second step, ubiquitin moiety is transferred to a ubiquitin-conjugating enzyme (E2). In the third step, mediated by a ubiquitin ligase or ligase complex (E3), ubiquitin is covalently attached to an NH₂ group of a lysine residue within the substrate protein (target protein). These reactions are reiterated using a specific lysine residue within the conjugated ubiquitin to generate a polyubiquitin chain. The 26S proteasome recognizes the chain configuration of ubiquitin and cleaves the peptide bonds in the targeted protein, while leaving the ubiquitin molecule intact for recycling. Both the conjugating enzyme (E2) and the ligase (E3) occur as members of large families, which determine substrate protein and tissue specificity.

In recent years, several ubiquitin-like (Ubl) molecules have been discovered in yeast and mammals. These proteins are conjugated to a lysine in the target proteins by a mechanism very similar to ubiquitin conjugation. However, a polyUbl chain is not generated. Examples are SUMO-1 (for small ubiquitin-related modifier, also known as PIC1/Ubl1/Sentrin and Smt3p in yeast) and RUB1 (for related to ubiquitin 1) family in yeast and *Arabidopsis* (and its counterpart NEDD8 in mammals). RUB1 in *Arabidopsis* is linked to protein degradation via auxin signaling (see text).

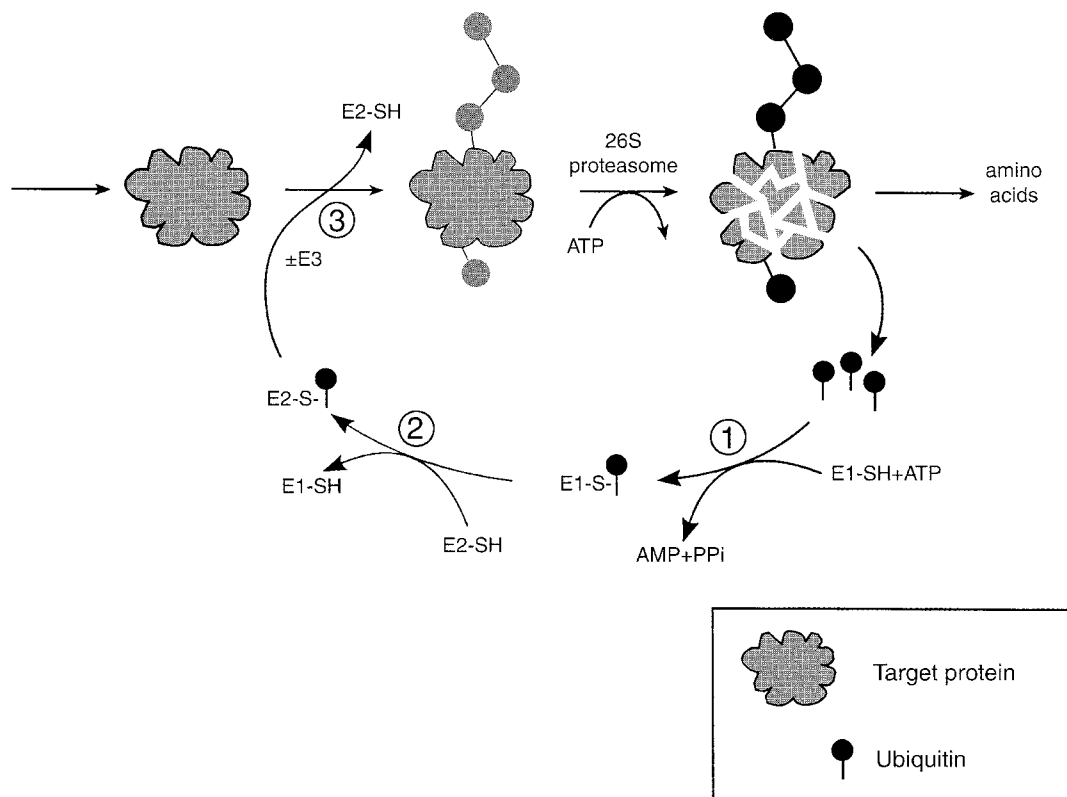


FIGURE 22-14 Diagram of the ubiquitin/26S proteasome proteolytic pathway. E1 and E2 are ubiquitin-activating and -conjugating enzymes, respectively, which catalyze steps 1 and 2 of the pathway. E3 is a ubiquitin-protein ligase which in step 3 ligates ubiquitin to the target protein. Each cycle of the pathway adds a ubiquitin molecule to the target protein resulting in a polyubiquitinated target protein which is recognized by the 26S proteasome. Reprinted with permission from Clough and Vierstra (1997), © Blackwell Science Ltd.

AXR1 is similar to only the N-terminal half of E1; it lacks the crucial C-terminal half and, thus, does not have E1 activity. A class of proteins with homology to the C-terminal half of E1, called ECR1 (for E1 C terminus related 1), has been discovered. The AXR1 and ECR1 combine *in vitro* to form a heterodimeric enzyme, which is able to activate the ubiquitin-like molecules of the RUB1 class, but not ubiquitin.

The RUB1 pathway is best characterized in yeast (*Saccharomyces cerevisiae*). In yeast, cell cycle progression is regulated by a ubiquitin ligase (E3) complex. The complex consists of a protein called Skp1, another protein called Cdc53 (a member of the cullin protein family), and one of a range of F box-containing proteins, such as Cdc4 or Grr1. Skp1 binds the F box proteins, Cdc53 is the protein to which Rub1 is conjugated, and the F box protein is responsible for recruiting the target protein into the ubiquitin–ligase complex. The complex is essential for cell cycle progression because in Skp1 mutants, both the cyclin–CDK inhibitor (Sic1p) and the G1 cyclins are stabilized, not degraded as required for cell cycle progres-

sion (for G1 cyclins and cyclin–CDK inhibitors, see Chapter 2). Thus, the Rub1 conjugation pathway converges with the ubiquitin–protein ligase (E3) complex (Fig. 22-15).

5.2. A Model for Auxin Signaling

In the last few years, *Arabidopsis* orthologs of the cullin protein family (AtCUL1), Skp1 proteins (ASK1, ASK2), and F box-containing proteins (TIR1) have been identified; and it has been confirmed that the three proteins interact to form a E3 ligase complex. Since loss-of-function mutations in *AXR1* and *TIR1* lead to reduced auxin sensitivity, it seems that auxin signaling in *Arabidopsis* operates through this pathway. Moreover, the AXR1–TIR1 pathway seems to be instrumental in the degradation of some protein(s) as part of auxin signaling. Genomic analysis indicates the presence of a small *AXR1* gene family in *Arabidopsis*, which suggests that different AXR1 isoforms may play roles in different auxin-mediated responses.

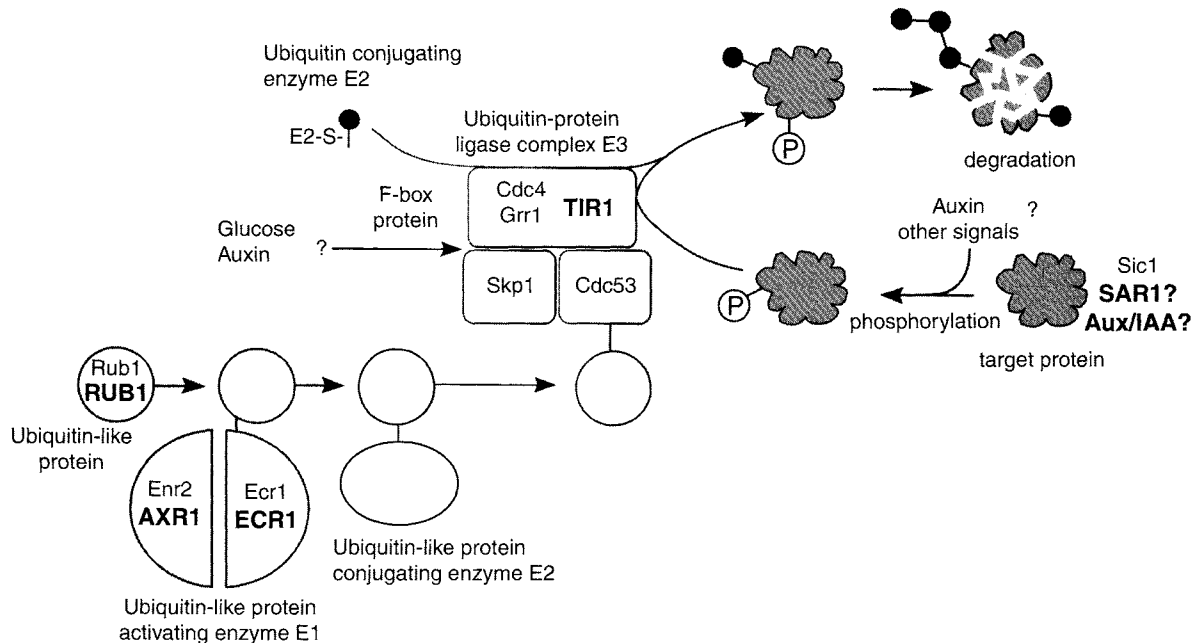


FIGURE 22-15 The ubiquitination pathway in yeast and *Arabidopsis*. *Arabidopsis* proteins are written in bold letters and are capitalized. In yeast, Skp1 binds to an F-box protein which in turn recruits the target protein (e.g., Sic1). The ability of proteins to be targeted depends on their phosphorylation status. Also, the efficient functioning of the ubiquitination pathway depends on the conjugation of the Rub1 to Cdc53, which is part of the complex with Skp1 and an F-box protein. In *Arabidopsis*, several components of this pathway have been identified, including RUB1, AXR1 and ECR1, and TIR1. The possible targets for proteolysis are Aux/IAA proteins and/or proteins that suppress auxin response (e.g., SAR1). The inducing signal for polyubiquitination in yeast could be glucose supply; in *Arabidopsis* it is shown to be auxin. From Leyser and Berleth (1999).

But which are the target proteins and where the auxin signal interacts with the degradation pathway are unknown. Two types of proteins have been proposed as possible targets for degradation: (i) Aux/IAA proteins, which are induced very rapidly after a supply of auxin and which have a short half-life, are one candidate. Many regulatory proteins with a short half-life (e.g., cyclins) carry sequences of amino acids that signal them for degradation by the proteolytic machinery of the cell. The Ps-IAA6 protein has been shown to carry a degradation signal in domain II near its N-terminal. That the degradation of Aux/IAA proteins may lead to an auxin response is supported by the observation that *Aux/IAA* genes cotransfected with auxin responsive promoter::reporter gene constructs in transient assays inhibit reporter gene expression. (ii) Some suppressor of *axr1* mutation, *sar1* mutants, are known from *Arabidopsis*. These mutants show reduced insensitivity to auxin in root growth (in other words, they are more like wild-type seedlings in the inhibition of root growth by 2,4-D). The nature of the protein encoded by the *SAR1* gene is unknown (see Fig. 22-15), but genetic analysis indicates that the *SAR1* protein acts downstream of *AXR1*. It has been suggested that it could be the repressor protein visualized for Aux/IAA-type genes. These alternatives must be kept open until more is known about the nature and roles of Aux/IAA proteins.

As to the possible site of interaction with the auxin signal, nothing can be said because the primary site of auxin perception remains unknown.

6. CHAPTER SUMMARY

An elucidation of the auxin signal perception-transduction pathway is one of the most intensively studied fields in plant biology and has occupied the attention of many laboratories in the world. Although considerable progress has been made since the mid-1980s, the goal of identifying an auxin receptor and the intermediate steps in the signal transduction pathway that lead ultimately to either the activation of H^+ -ATPases in the plasma membrane or the transcription of primary auxin responsive genes remains elusive.

Hormone-binding studies have identified an auxin-binding protein from maize coleoptiles that has the hallmarks of a receptor. Antibodies against the protein inhibit electrical phenomena both in mesophyll protoplasts and in stomatal guard cells, and the protein has been localized on the external face of the plasma membrane, even though it is located predominantly inside the endoplasmic reticulum. Recent molecular work has

confirmed that ABP1 has a significant role in auxin-induced cell enlargement and possibly in cell division. Similar proteins are known from a variety of other monocots and dicots, but doubts remain whether this protein is an auxin receptor or an exclusive auxin receptor. Experiments using phytochemicals that block IAA efflux from cells indicate that cell growth in stem segments is correlated more closely with the intracellular concentration of IAA than with extracellular IAA. Mutants that are defective in auxin uptake show slowed responses to auxin, which means that at least one site for auxin perception is intracellular. Some soluble auxin-binding proteins have been reported and it might be worth while to investigate them further.

Molecular and genetic studies on auxin signaling have identified elements that act at later steps in auxin signal transduction. Several classes of primary auxin response genes are known, among which the *Aux/IAA*-type genes from *Arabidopsis* and pea are better investigated. These genes encode short-lived nuclear proteins that have the hallmarks of transcription factors, but they seem to act in concert with other transcription factors, known as auxin response factors, which bind to auxin response elements in primary auxin response genes. *In vitro* analyses show that multiple combinations of these regulatory proteins as homo- and heterodimers are possible. Such combinations of transcription factors with different *cis* elements may underlie the differential activation of auxin responsive genes in cells and tissues. Although the functions of *Aux/IAA*-type genes are not known, both *Aux/IAA*- and *ARF*-type proteins are important in auxin signaling because mutations in them cause severe abnormalities in plant development and tropic responses. Unfortunately, the downstream target genes of *Aux/IAA*- and *ARF*-type proteins or the upstream elements that connect them to auxin perception are unknown. Attempts are being made using suppressor screens, as well as targeted mutagenesis, to identify elements that interact with them.

An analysis of auxin-insensitive mutants indicates that a cardinal step in auxin signaling involves proteolysis or modification of some protein or proteins *via* a modified ubiquitin pathway. The major components of this degradation pathway in *Arabidopsis*, the *AXR1*-*TIR1* pathway, are in place, but the targets of degradation are unknown. Also unknown are the connections between auxin signaling and the degradation pathway.

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Absciscic Acid Signal Perception and Transduction

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1. ABSCISIC ACID (ABA)-INDUCED RESPONSES

As discussed in Chapters 16 and 18, ABA mediates many stress-related responses in vegetative organs caused by dehydration, high salinity, cold, wounding, and attack by pathogens; it also plays a central role in several events in mid- to late embryogenesis in seed

tissues, including desiccation tolerance, imposition and maintenance of seed dormancy, and synthesis of some storage proteins. Many of these responses, such as the synthesis of compatible osmolytes (e.g., proline), defense-related proteins (e.g., superoxide dismutase), and late embryogenesis abundant (LEA) proteins, involve regulation of gene expression, whereas others, for instance, stomatal closure during water deficit, involve only cell volume changes, not regulation of gene activity. Still other responses, such as inhibition of seed germination or root growth probably, are regulated by alterations in water balance, but may well involve changes in gene expression (Fig 23-1).

2. PERCEPTION OF ABA SIGNAL

Induction of genes and stomatal closure both require perception of the ABA signal. However, despite considerable effort, no protein has been identified as yet that qualifies as an ABA receptor. Moreover, there is the possibility that there may be several ABA receptors. The site of perception of the ABA signal is also unclear. There is evidence for an extracellular perception of ABA at the surface of the plasma membrane and, also, at intracellular sites.

2.1. ABA-Binding Proteins and Site(s) of Perception

In the 1970s and early 80s, binding studies using radiolabeled ABA had demonstrated some high affinity binding sites in subcellular fractions from leaves of

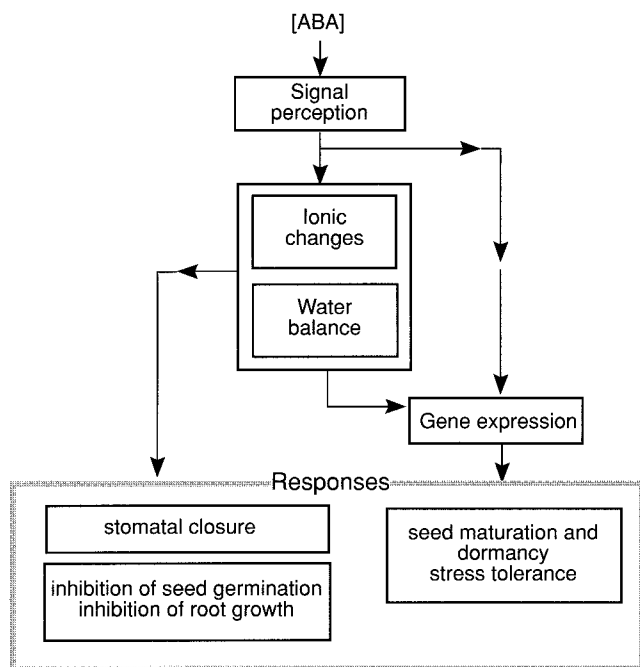


FIGURE 23-1 An overview of ABA-induced responses. Inhibition of seed germination and root growth are shown with stomatal regulation, but it is not known whether they involve changes in water relations only or gene expression. Modified from Hetherington and Quatrano (1991).

Vicia faba, but there has been little subsequent work along these lines and this work is not reviewed here.

In more recent years, several studies, using both guard cells and stomatal behavior and isolated protoplasts and gene induction as test systems, have suggested an extracellular site for ABA perception. For stomata of *Commelina communis*, it was shown that ABA-induced closure was brought about if ABA was present in the bathing medium, but not if it was injected into the guard cells, which suggested that ABA perception occurred at the outer surface of the plasmalemma. A similar conclusion was reached for ABA perception in barley aleurone protoplasts. Evidence for a surface perception of ABA has also come from the use of impermeant ABA, i.e., ABA linked to bovine serum albumin (BSA). Such impermeant ABA was still able to induce the expression of a fusion construct, consisting of the promoter of the wheat *EM* gene linked to the reporter gene, β -glucuronidase (*GUS*), in rice suspension cell protoplasts in a dose-dependent manner.

Other studies, however, suggest that intracellular sites for ABA perception also occur. Caged ABA, microinjected into the guard cells and released intracellularly, can cause stomatal closing. It has also been argued that the pH of the bathing medium has import-

ant consequences. At mildly acidic extracellular pH (pH 4.5–6.0), ABA in protonated form would diffuse into the cells; also, some carrier-mediated uptake of the ABA anion (ABA^-) may occur (see Chapter 13). Such movements would raise the intracellular concentration of ABA and bring about stomatal closure.

2.2. ABA Stereoisomers and Biological Activity

Suzanne Abrams and colleagues at NRC, Plant Biotechnology Laboratory, Saskatoon, Canada, have synthesized several structural isomers of natural ABA [*S*-(+)-ABA], including the (*R*)-enantiomer, derivatives such as ABA aldehyde, ABA alcohol, and metabolites such as 7'-hydroxy ABA, and studied their uptake and biological activities. Many of these isomers and derivatives, which apparently cannot be converted to the natural ABA *in vivo*, have equal, or greater, activity than natural ABA in many bioassays (e.g., inhibition of seed germination in wheat, induction of oleosin and napin genes in *Brassica napus* embryos, induction of *LEA* genes in somatic embryos of wheat and spruce, reversal of GA-induced α -amylase gene expression in barley aleurone layers). These data suggest either that more than one type of ABA receptor exists or that the receptor is able to accept structural variations on the natural hormone within a certain range.

3. ABA AND STOMATAL BEHAVIOR

Stomata play a critical role in regulating the supply of atmospheric CO_2 for photosynthesis and in preventing an excessive water loss *via* transpiration. Guard cells regulate the opening and closing of the stomatal pore and maintain this delicate balance, i.e., not to starve the plant for CO_2 by restricting the influx of CO_2 nor desiccate the plant by allowing excessive water loss. Not surprisingly, stomatal pore opening or closing is regulated by a variety of signals: CO_2 , humidity (not relative humidity), red and blue light, and, among plant hormones, ABA, and auxins. ABA is one of the important and better investigated signals, but at least some signals, such as humidity, have been shown to regulate stomatal behavior independently of ABA. These multiple environmental and endogenous signals, at times contradictory, are sensed and integrated into a single coherent response by guard cells.

3.1. Mechanics of Stomatal Opening and Closing

Stomata open because of a rise in osmotic pressure (OP) of guard cell vacuoles, which is due to an influx of K^+ and anions such as Cl^- from the neighboring epidermal cells (Fig. 23-2). Organic solutes, such as sucrose and malate, also contribute to the rise in osmotic pressure. Accumulation of these solutes in the vacuole results in an influx of water and a consequent volume change in the guard cells. The wall architecture of guard cells is such that ballooning out of the thin outer wall pulls with it the thicker inner wall (bordering the pore), thus opening the pore (see Fig. 16-8). During stomatal closure, K^+ and anions (and other solutes) move out of the cell or to intracellular compartments, which results in loss of water and closure of the pore.

3.2. The Plasmalemma of Guard Cells Is Rich in Ion Channels

Stomatal opening and closing involve rapid volume changes of guard cells. Commensurate with that function, the plasma membrane of guard cells, in contrast to that of mesophyll cells, is rich in ion channels. Anion channels, both inward- and outward-bound K^+ channels, Ca^{2+} channels, and ATPase-driven H^+ or Ca^{2+} pumps (for these channels and pumps, see Box 1 Chapter 13; also Fig. 25-17 in Chapter 25). In guard cells, the vacuolar membrane, although very important, has not been studied to the same extent as the plasma membrane; hence, in the following account, focus is on the plasma membrane.

3.3. Role of ABA in Stomatal Behavior

That ABA regulates guard cell behavior is shown in several ways: (i) Epidermal peels are obtained easily from leaves of some plants, such as fava bean (*Vicia faba*) and day flower (*Commelina communis*). If such peels are placed in ABA solutions, they show a closure of stomata. (ii) ABA can be packaged inside chemical baskets (molecular cages), microinjected into a cell, and released there. ABA introduced into guard cells of *Commelina* in this manner has been shown to cause stomatal closure. (iii) More direct evidence comes from ABA-deficient mutants—they are unable to close their stomata unless supplied with exogenous ABA (see Chapters 10 and 16). ABA response mutants, *abi1-1* and *abi2-1* (see Section 5), are also unable to close their stomata because of a defect in ABA signaling. Exogenous ABA has no effect on these mutants. (iv) In an ingenious experiment, a gene encoding an anti-ABA antibody was expressed in transgenic tobacco plants.

The antibody effectively reduced the endogenous ABA content, and the transgenic tobacco plants were unable to close their stomata when subjected to water stress.

3.3.1. ABA Brings about Stomatal Closure by Modulating the Activities of Some Ion Channels

Electrophysiological measurements indicate that ABA treatment results in a depolarization of the plasma membrane. This depolarization results from an activation of the slow (S-type) anion channel, the channel responsible for the slow but sustained efflux of anions over a wide range of voltage, as well as inhibition of the inward-directed K^+ channel. It probably also inactivates the plasma membrane H^+ -ATPase. Depolarization provides the driving force for K^+ efflux through voltage-activated K^+ channels. As levels of K^+ (and malate) drop, guard cells release water, relaxing the outward flex of the outer wall and closing the pore.

3.3.2. Calcium Is Involved in ABA Regulation of Ion Channels

Epidermal peels put in ABA solution show a closure of stomata and an efflux of K^+ and anions. It can be shown further that if peels are taken out, say after 2 min, and ABA is washed off, the efflux of K^+ (or ^{86}Rb) and anions continues for 20 or more min, suggesting that some secondary messengers are involved. The secondary messenger is calcium. Calcium can also be caged, like ABA, inside chemical baskets, microinjected into the guard cell, and released when inside. The resultant increase in the free cytosolic $[Ca^{2+}]$ leads to an efflux of anions and K^+ and closure of stomata independently of ABA treatment. Also, caged ligands for receptor-activated Ca^{2+} channels can be taken into the cell and released, leading to a release of stored Ca^{2+} and closure of stomata. Thus, there is ample evidence that rise in cytosolic Ca^{2+} is involved in pore closure.

Cytosolic Ca^{2+} levels rise by the movement of Ca^{2+} from outside and/or by its release from internal reserves. In plant cells, the largest amounts of calcium are present in the cell wall, but the internal reserves of Ca^{2+} are mostly in the vacuole and, to a lesser extent, in the endoplasmic reticulum (ER). The movement of Ca^{2+} from outside occurs via nonselective channels. The release from internal reserves occurs through receptor-activated Ca^{2+} channels on the vacuolar and/or ER membranes (see Chapter 25). The rise in cytosolic $[Ca^{2+}]$ activates the slow anion channel and inhibits the inward-directed K^+ channel. Activation of the slow anion channel brings about a depolarization of the membrane potential into the range at which the outward K^+ channel is opened. The combination of an efflux of anions and K^+ leads to stomatal pore closure (Fig. 23-3).

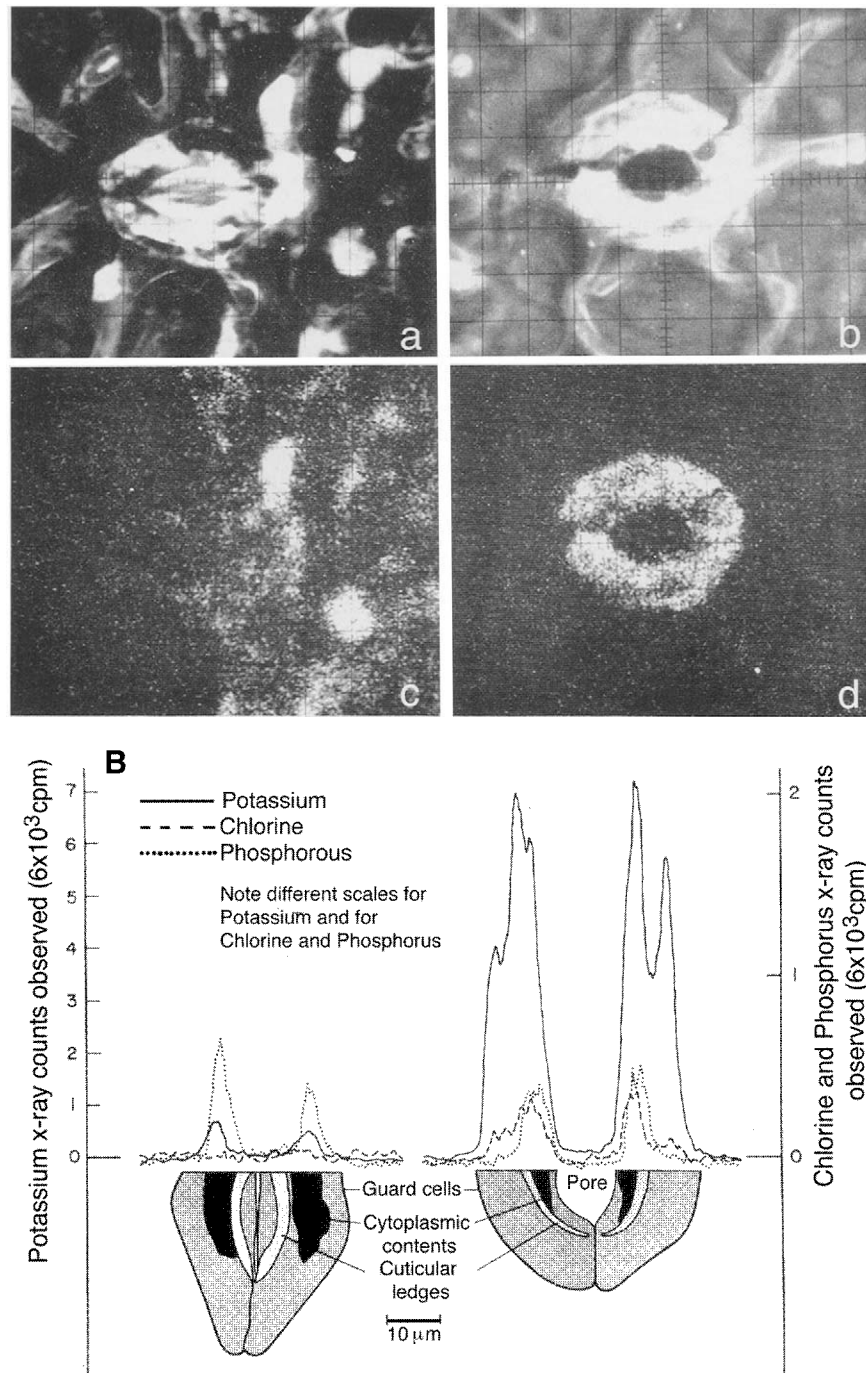


FIGURE 23-2 A stomatal complex in *Vicia faba* and elemental distribution in guard and subsidiary cells. (A) A stomatal complex with closed (a) and open (b) pores and distribution of potassium ions in subsidiary and guard cells of the same (c and d). (c and d) Movement of K^+ from subsidiary cells into guard cells. Such movement results in uptake of water and opening of stomatal pore; the pore is closed on efflux of K^+ . For these photographs, epidermal strips were quick frozen, freeze-dried, given a light coat of carbon to improve electrical conductivity, and examined under an electron probe microanalyzer. (a and b) Secondary electron images as in a scanning electron microscope. Different elements in the sample emit characteristic X rays when scanned by the electron beam; the x rays can be sorted according to their energies and used to indicate the presence or absence of a specific element in the sample. (c and d) Concentration and distribution of K^+ determined from X rays. (B) Elemental scan for potassium, chlorine, and phosphorus for guard cells in closed and open states. The guard cells are the same as in A. From Humble and Raschke (1971).

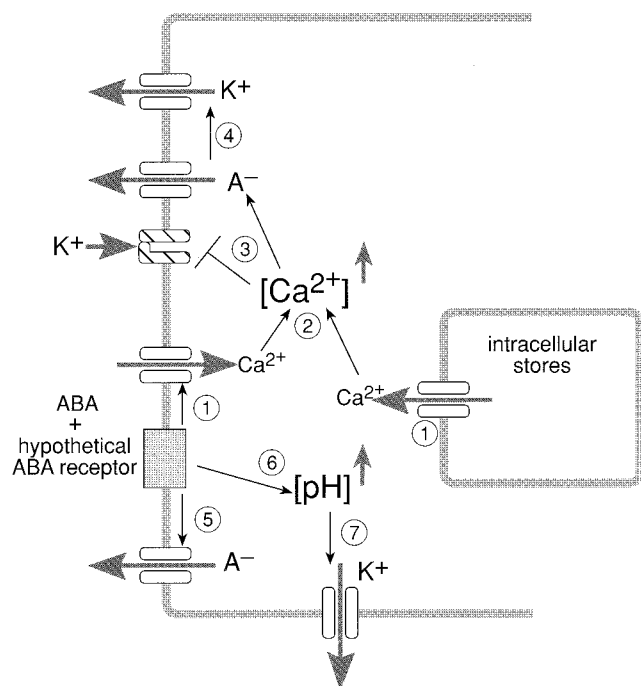


FIGURE 23-3 A schematic model showing the likely chain of events in stomatal pore closure following ABA treatment. ABA is shown to bind to a hypothetical receptor on the plasma membrane. Such binding causes entry of Ca^{2+} from extracellular sources and/or release from intracellular stores (1). The rise in cytosolic Ca^{2+} (2) causes an inhibition of the inward-directed K^+ channel and activation of the slow anion channel (3). The resultant depolarization of the plasma membrane opens the outward-directed K^+ channel (4). The efflux of anions and K^+ results in pore closure. In addition, there is a further enhancement of K^+ efflux by ABA, which is independent of calcium. ABA is thought to induce the opening of the anion channel (5) and inhibit H^+ -ATPase, causing an increase in the cytosolic pH (6). The increase in cytosolic pH causes further depolarization of the membrane potential and activates the outward-directed K^+ channel (7), causing stomatal pore closure. Based on data from Blatt and Thiel (1993).

Although ABA triggers stomatal closure, a rise in free Ca^{2+} levels is not always correlated with ABA-induced stomatal closure. Stomata can close in response to ABA without any measurable increase in cytosolic $[\text{Ca}^{2+}]$. Thus, other possibilities have been suggested. ABA is thought to cause an increase in cytosolic pH by inhibiting the activity of H^+ -ATPase. The resultant increase in cytosolic pH causes depolarization of the plasma membrane to the level that activates the outward-directed K^+ channel, a channel that is independent of Ca^{2+} levels.

3.4. Section Summary

ABA is one of the major signals that regulate stomatal behavior. The plasmalemma of guard cells is rich in

ion channels and pumps. ABA brings about loss of turgor in guard cells and stomatal pore closure by increasing the cytosolic content of free calcium ions, which enhances the activity of slow anion channels. The resultant depolarization of the plasma membrane leads to opening of the voltage-gated, outward-directed K^+ channels. ABA also inhibits the inward-directed K^+ channel. As a result, K^+ and organic solutes such as malate and anions such as Cl^- leave the cell, causing water to move out and the stomatal pore to close. In addition, there is an enhancement of K^+ efflux by an ABA-induced increase in cytosolic pH. Such an increase in cytosolic pH results in further depolarization of the plasma membrane, opening of K^+ efflux channels, and pore closure.

4. ABA-REGULATED GENE EXPRESSION

Since the first report of an ABA-induced gene expression in the late 1980s, hundreds of genes have been reported to be induced by ABA. However, among these genes, those that have been shown to be regulated at the transcriptional level are much fewer in number.

Functional analyses of promoter regions of these genes have yielded several *cis* sequences that are important for induction by ABA.

4.1. Functionally Important *cis* Sequences

4.1.1. ACGT Core-Containing Elements

One sequence of 8 to 10bp, known as the ABA response element (ABRE), carries a core sequence of four nucleotides, ACGT. ACGT core-containing ABREs are known from promoters of various ABA-regulated genes in seed tissues (e.g., *LEA* genes, some storage protein and oleosin genes) and stress- and ABA-induced genes in vegetative tissues. One to several ABREs may occur in the promoter sequence of a gene; e.g., two ABREs occur in the promoter of the *EARLY METHIONINE (EM)* gene in wheat (see Table 23-1), which encodes a *LEA* D19-type protein.

The ACGT core occurs not only in ABREs, but also in a motif known as the G box, which is found in promoters of several other genes. These genes are not induced by ABA, but by various environmental stimuli, such as white light, UV, and anaerobiosis, as well as phenolic compounds, such as coumaric acid. For instance, the G box motif occurs in genes associated with photosynthesis, which are induced by white light, such as the *RBCS* gene encoding the small subunit of

TABLE 23-1 Some *cis*-Acting Promoter Elements Responsive to ABA^a

Species	Gene	Element	Sequence ^b
<i>Triticum aestivum</i> (wheat)	EM	Em1a	-AC ACGT GGC-
		Em1b	-AC ACGT GCC-
<i>Oryza sativa</i> (rice)	RAB16	Motif I	-GT ACGT GGC-
<i>Hordeum vulgare</i> (barley)	HVA22	ABRE3	-GCC ACGT TACA-
	HVA1	ABRE2	-CCT ACGT GGC-
<i>Zea mays</i> (maize)	C1	Sph	-CGTGTCTGTCATGCAT-
<i>Craterostigma plantagineum</i> (resurrection plant)	CDeT27-45	—	-AAGCCCAAAATTTCAC
		—	AGCCCCGATAACCG-
<i>Arabidopsis thaliana</i>	RD22	Myb	-YAAC(G/T)G ^e -
		Myc	-CANNTG ^f -
<i>A. thaliana</i>	RD29A	ABRE	-T ACGT GTC-
		DRE ^c	-TACCGACAT-
<i>Daucus carota</i>	DC3	TT motif	-TTTCGTGT-
<i>Zea mays</i>	RAB17	DRE1 ^d	-CGAGAAGAACCGA
			GA-
<i>Z. mays</i>	RAB17	DRE2 ^d	-CCGGGCCACCGACG
			CACGG-

^aCompiled from Leung and Giraudat (1998), Yamaguchi-Shinozaki and Shinozaki (1994), Busk and Pagés (1997), and Kim *et al.* (1997).

^bThe ACGT core in ABREs is in bold letters.

^cThe DRE from *Arabidopsis* is stress inducible but not ABA inducible.

^dDRE1 and DRE2 from maize are ABA inducible and were named on the basis of the sequence similarity to DRE from *Arabidopsis*.

^eY, pyrimidine.

^fN, any nucleotide.

RUBISCO and the *CHLA/B* gene encoding chlorophyll a and b-binding proteins. It also occurs in the *ADH1* gene in maize that encodes alcohol dehydrogenase, which is induced during anaerobic respiration. Thus, the ABRE and G box are subsets of ACGT-containing *cis* elements, and an ABRE is defined by function, i.e., it is induced by ABA.

The various ABREs and G boxes differ from one another in the bases flanking the core ACGT, but a clear distinction between them has not been possible. The variable sequences probably specify the particular transcription factor that binds to the ABRE, or the G-box, and thus provide the necessary discrimination between different signals (see Section 4.1.3).

4.1.2. Not All ABA Responsive Elements Have an ACGT Core

Although ACGT core-containing *cis* elements are specific for ABA-inducible genes, not all genes that are induced by ABA carry a *cis* element with an ACGT core. The C1 gene in maize is a regulatory gene whose product modulates the transcription of

several other genes that encode enzymes in anthocyanin biosynthesis during kernel development/maturation. The C1 gene is regulated by ABA (also by VP1, see later) and carries a *cis* element known as Sph that confers ABA inducibility (see Table 23-1). Many stress- and ABA-inducible genes that are expressed in vegetative tissues also do not show an ABRE with an ACGT core (e.g., RD22 gene in *Arabidopsis* and CDeT27-45 genes in the resurrection plant, *Craterostigma plantagineum*). Still other genes may show two response elements: one specific to stress and other to ABA (e.g., *Arabidopsis* RD29A gene) (Table 23-1, see also Section 4.4).

4.1.3. ABA Response Complexes

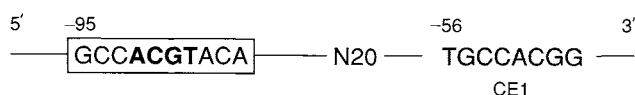
As explained in Appendix 1, individual response elements require other *cis* elements to couple hormone effects to the basal transcriptional machinery, i.e., *in vivo*, they function within the context of a response complex. For ABA-regulated genes, the coupling element may be an ACGT core-containing element (Fig. 23-4). Also, the relative positions of the coupling

elements, i.e., whether upstream or downstream, with respect to the ABRE vary. They may be contiguous (or partially overlapping) or separated by a variable number of nucleotides, but their relative positions and orientations are important for ABA inducibility. The nucleotide sequences of the two elements, as well as their relative positions with respect to each other, determine the types of transcription factors that bind to these sequences. In some cases, three or more elements may be required for tissue organ specificity.

4.2. ABA-Specific Transcription Factors

Several transcription factors for ABA-induced genes have been isolated, both for seed-specific genes and those that are expressed in vegetative tissues during stress. Among the seed-specific factors, EMBP1 (for *EM* gene binding protein 1) and TAF1 (for transcription-associated factor 1) proteins, which bind to promoter sequences in wheat *EM1* and rice *RAB16* genes, respectively, were the first to be isolated. Homologues of EMBP1 have been isolated from other plants, including rice (*Os-BZ8*). Two transcription factors, which bind to promoter sequences in the *DC3* gene (a *LEA* gene) in carrot, which is expressed in zygotic and somatic embryos, have been isolated. All of these transcription factors belong to the basic domain leucine zipper (bZIP) family, which shows varying affinities for *cis* elements with an ACGT core, including the ABREs and G boxes present in seed-specific or environmentally induced promoters, respectively. bZIP proteins bind to DNA as homodimers as well as heterodimers.

HVA22



HVA1



FIGURE 23-4 Schematic illustrations of two ABA-specific response complexes in barley (*Hordeum vulgare*). In the promoter complex for *HVA22*, the coupling element occurs 3' to the ACGT-containing ABRE and is separated by 20 nucleotides (N), whereas in *HVA1*, the coupling element occurs 5' to the ABRE and is adjacent to it. Adapted from Shen and Ho (1997).

Some other families of transcription factors also regulate ABA responsive genes. The C1 protein in maize belongs to the MYB family. As mentioned earlier, *RD22* is an *Arabidopsis* gene that is induced by both dehydration and ABA. The promoter of *RD22* does not contain an ABRE with an ACGT core; instead, as shown in Table 23-1, it contains two *cis* sequences believed to be recognized by two different transcription factors. One factor, *RD22BP1*, belongs to a basic region helix-loop-helix family and binds to the Myc sequence; the other, *At-MYB2*, belongs to the MYB family and binds to the Myb sequence. Still another transcription factor in *Arabidopsis*, *At-HB7*, which is induced by both ABA and stress, belongs to the family of homeodomain-containing proteins.

4.2.1. Multiple Proteins Bind to *cis* Sequences

Because some ABA-induced genes are also induced by environmental factors, such as osmotic stress or cold, and because these genes may be expressed differentially in different tissues (see Chapter 16), it is expected that the promoters of these genes would show multiple *cis* sequences for binding by different proteins. Which proteins actually bind would be determined by the signal and/or the tissue. The *RAB17* gene in maize, a *LEA D-11* (dehydrin) class, is induced in late embryogenesis and can be induced in embryonic or vegetative tissues by either ABA or desiccation. The promoter of *RAB17* contains as many as nine *cis* elements, as shown by *in vivo* footprinting assays.

4.2.2. Induction of Genes Encoding Transcription Factors

Genes encoding transcription factors are often induced by the hormone in question and are also primary response genes, i.e., they do not require prior synthesis of some other protein. Among the transcription factors mediating ABA-induced gene expression, some have been shown to be induced by ABA (e.g., *At-HB7* in *Arabidopsis*, *OsBZ8* in rice) or to be encoded by primary response genes (e.g., *RD22BP1* in *Arabidopsis*).

4.3. Seed-Specific Transcription Activators

In addition to the transcription factors mentioned in Section 4.2, other transcription factors regulate ABA-inducible genes in an organ-specific manner. Two transcription factors that have been very well investigated are maize *VP1* and *Arabidopsis* *ABI3*. The genes for these factors, *VIVIPAROUS1* (*VP1*) in maize and *ABSCISIC ACID INSENSITIVE3* (*ABI3*) in *Arabidopsis*, were isolated following analysis of two ABA response

mutants, *vp1* and *abi3*, which showed insensitivity to exogenous ABA in inhibiting seed germination (see Section 5). However, unlike other response mutants, the effects of these mutations were noted primarily in seed tissues. Detailed analysis of the mutants and cloning of their wild-type genes confirmed that these genes were expressed mainly in seed tissues there is

(some evidence that *ABI3* may also be expressed in vegetative tissues). What came as a surprise was that the genes encoded proteins that acted as transcriptional regulators, i.e., they modified the activities of other transcription factors in a stage-specific manner during seed development/maturation. Box 23-1 provides background information on this subsection.

BOX 23-1 SEED MATURATION PROGRAM IN MAIZE

THE SEED DEVELOPMENT/MATURATION program in maize is regulated by ABA, light, and some seed-specific regulatory proteins in a complex manner. Wild-type maize kernels develop a purple color during mid- to late stages of seed development/maturation. The color is due to the synthesis of anthocyanins in the aleurone layer, the outer layer of the endosperm, as well as in the scutellum of the embryo. The synthesis of anthocyanins involves activation of many structural genes (e.g., *A1*, *A2*, *C2*, *Bronze1*, *Bronze2*), which are under the control of a few regulatory genes, such as *C1*. The *C1* gene, in turn, is regulated by the protein VP1 and ABA. In *vp1* mutants, *C1* is not expressed with the result that the kernels do not develop the purple color and appear colorless. In addition, *vp1* mutants fail to undergo the maturation program typical of wild-type seeds; instead, they enter the germination program, which is characterized by vivipary (emergence of coleoptile and roots from the kernel while still on the cob) and activation of enzymes involved in the hydrolysis of stored food products, such as α -amylase. The combination of germinated kernels that lack the purple pigments distinguishes *vp1* mutants from wild-type seeds that are both nonviviparous and purple (Fig. 23-5). Other *vp* mutants of maize are ABA synthesis mutants (e.g., *vp2*, *vp3*, *vp5*); they also are viviparous, but develop the purple pigments. Their viviparous phenotype can be restored to wild type by treatment with exogenous ABA.



FIGURE 23-5 Phenotypic effect of *vp1* mutation on maize seed development. A portion of maize ear segregating the *vp1* mutant is shown. Wild-type kernels have anthocyanin pigment (they appear purple) and are dormant. Mutant kernels are viviparous and lack anthocyanin pigment. They appear yellow and show embryo growth. Reprinted with permission from McCarty *et al.* (1991), © Cell Press.

Light also plays a role in activating C1. Wild-type maize ears that develop in total darkness produce colorless seeds. The light regulation of C1 is highly complex and is not covered here.

4.3.1. VP1 and ABI3 Modulate the Expression of Seed-Specific ABA-Regulated Genes and Some Other Genes

As explained in Chapter 18, seed development/maturation is characterized by a coordinated expression of sets of genes in a time-specific manner, and ABA regulates the expression of many of these genes during mid-to late embryogenesis. These genes include LEA-type genes whose products seem to be involved in desiccation tolerance and many other genes whose products confer tolerance to heat shock, oxidative damage, and some storage protein and oleosin genes. In *abi3* or *vp1* mutants, mRNAs of these genes fail to accumulate. Figure 23-6 shows the effects of *aba1* (an ABA biosynthesis mutant) and *abi3* mutation on the accumulation of some representative mRNAs during silique development in *Arabidopsis*. The *aba1* mutant has only about 5% of the wild-type content of endogenous ABA, but still is able to show substantial amounts of *Arabidopsis* homologues of CRUCIFERIN1 and NAPIN3 (*CRC* and *At2S3*) gene transcripts, but lesser amounts of *AtEM1* and *AtEM6* transcripts. The *abi3-4* mutant did not show any transcripts except some *CRC*.

In addition to regulating ABA-induced genes in seed tissues, VP1 and ABI3 proteins also regulate some genes on their own. Thus, their spectrum of activity extends beyond ABA regulation.

In normal development, VP1 and ABI3 are expressed exclusively in seed tissues. However, if their coding sequences under a strong constitutive pro-

motor are expressed in transgenic plants or cells in suspension culture, the corresponding proteins can accumulate ectopically in vegetative parts. When so expressed, they can drive the expression of many seed-specific genes in vegetative parts with or without exogenous ABA (Fig. 23-7).

4.3.2. Structure of VP1 and ABI3 Proteins

VP1/ABI3 proteins are functional homologues. Genes encoding similar proteins have been cloned from a number of monocots and dicots (e.g., rice, barley, wild oat, bean, poplar). The deduced sizes of proteins vary (VP1 is 691 amino acid long, its counterpart in bean is 752 amino acids), but in all cases, the proteins share a common structure (Fig. 23-8). They have four conserved domains: domain A1 located in the N-terminal acidic region and three basic domains, called B1, B2, and B3, toward the C-terminal. The proteins show no specific DNA-binding motifs, but, nonetheless, bind DNA and, also, are believed to affect the activities of other transcription factors by protein-protein interactions. The A1 domain has a function in transcription activation; the functions of basic domains are still not clear but B3 and B2 are thought to be involved in DNA binding (see later).

The discovery of VP1 and ABI3 has allowed a dissection of organ-specific expression of genes and how developmental pathways may be regulated. In the following, the action of VP1 is highlighted. ABI3 probably acts in a similar manner.

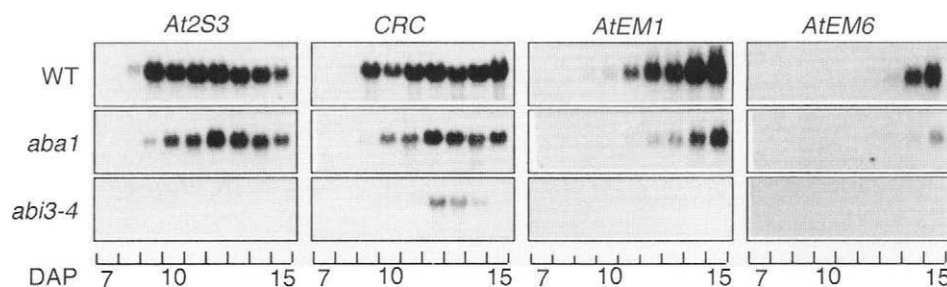


FIGURE 23-6 Comparative gel blot analysis of the expression pattern of representative mRNAs during silique development in wild type (WT), *aba1* (an ABA biosynthesis mutant), and *abi3-4* mutants. Each of the individual gene-specific probes was hybridized to total RNA (5 μ g) isolated from two siliques of each type of plant at days indicated, from 7 days after pollination (DAP) to 15/16 DAP when siliques in all cases were dry and ripe. *CRC* and *At2S3* encode *Arabidopsis* homologues of CRUCIFERIN 1 and NAPIN3 storage proteins in *Brassica*, and *AtEM1* and *AtEM6* encode *Arabidopsis* homologues of wheat EM protein, a LEA protein expressed in late embryogenesis. From Parcy *et al.* (1994).

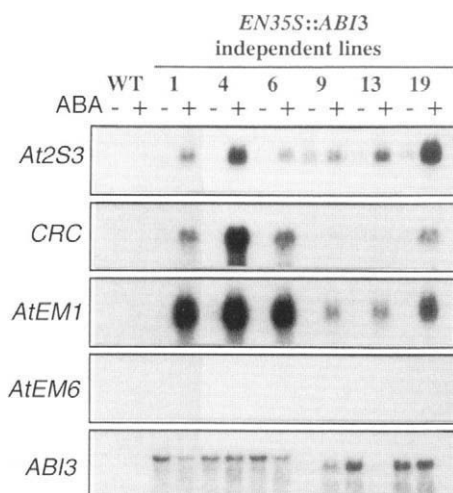


FIGURE 23-7 Ectopic expression of some seed-specific genes in *Arabidopsis* plants transformed with a construct consisting of the *ABI3* coding sequence under the control of a strong constitutive promoter (35S CaMV). Results from the wild type (WT) and six different transformant lines (1–19) are shown. The indicated gene-specific probes were hybridized to total RNA (5 µg) from wild type and transformed plants. Seeds were germinated and grown for 11 days *in vitro* in the absence of ABA and where then transferred for an additional 2 days on plates with (+) or without (–) 50 µM ABA. Note: In the bottom row, *ABI3* transcripts were of two different sizes in different transformants and, for unexplained reasons, were missing in lanes 9 and 13. From Parcy *et al.* (1994).

4.3.3. Mode of Action of VP1

How does VP1 affect the expression of seed-specific genes? Is it essential for the induction of ABA-regulated genes in seed tissues and does it interact with the same *cis* elements as those involved in ABA-induced gene expression? Several seed-specific genes have been studied. The two most studied are the *EM* gene in wheat (or its homologues in rice and maize) and the *C1* gene, one of the key regulatory genes in anthocyanin biosynthesis in maize kernels.

4.3.3.1. VP1 Is Not Essential for Induction of Genes

The answer to the first question seems to be in the negative. In mutants with a defective VP1 protein, seed-specific genes, such as *EM* and *RAB28*, can still be induced by high concentrations of exogenous ABA.

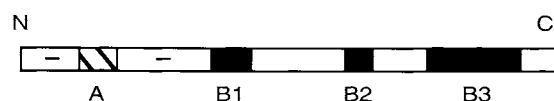


FIGURE 23-8 Schematic diagram of VP1/ABI3-like proteins. The proteins have an N-terminal acidic region (---), which contains domain A1, followed by three basic domains, B1, B2, and B3, in that order, toward the C-terminal.

In vitro -binding assays show that EMBP1 is able to bind to DNA segments with an ABRE, but the binding is enhanced in the presence of VP1. Similarly, *in vivo* footprinting assays show that nuclear proteins, whose identity is unknown, bind to ABREs in the *RAB28* promoter and that such binding is still seen in a *vp1* mutant background. These data suggest that VP1 is not essential for binding of transcription factors such as EMBP1 to ABREs *in vitro* or *in vivo*, but that such binding is much tighter in the presence of VP1.

4.3.3.2. VP1 Binds Cooperatively to a Transcription Factor for the EM Gene

Another set of experiments shows that VP1 interacts with the same ABREs as those involved in ABA-induced gene expression. To show this interaction, two constructs were prepared; one consisting of a reporter gene coupled to deletion segments of the *EM* promoter and the other consisting of the *VP1* coding sequence under the control of a constitutive promoter. The reporter gene construct was transfected into maize protoplasts in suspension culture for transient expression with or without the *VP1* construct and with or without ABA. Under these conditions, expression of a reporter gene could point out accurately which segments of the *EM* promoter interacted with VP1 and whether VP1 acted independently of ABA. Data presented in Fig. 23-9 indicate that VP1 activates the *EM* promoter principally by an Em1a-like ABRE. Partial deletion in this *cis* element gave a much reduced expression of the reporter gene. As shown earlier, the same motif is also activated by ABA (see Table 23-1).

Since both VP1 and ABA activate the same *cis* motif and, moreover, have a synergistic effect, it is reasonable to assume that VP1 acts cooperatively to enhance the efficacy of the transcription factor that binds to the Em1a motif. Since the VP1 protein itself has no obvious DNA-binding domain, it is proposed that it binds to a transcription factor, which, in turn, binds to the ABRE (Fig. 23-10). In an effort to find this transcription factor, the yeast two hybrid screen was utilized using rice orthologs of EmBP1 of wheat and VP1 of maize (the conserved regions of *OsBZ8* were fused to the conserved B1 and B2 domains of VP1 and used to screen a rice cDNA library; for the yeast two hybrid screen methodology, see Appendix 1). A cDNA clone encoding a bZIP transcription factor, TRAB1 (for transcription factor responsible for ABA regulation) was isolated and shown to interact with both VP1 and ABREs (in various promoters) and to mediate ABA-induced gene expression. TRAB1 is expressed in most plant parts and cells in culture and the expression of its gene is highly induced by ABA. The acidic domain of VP1 is an activator domain, because deletion

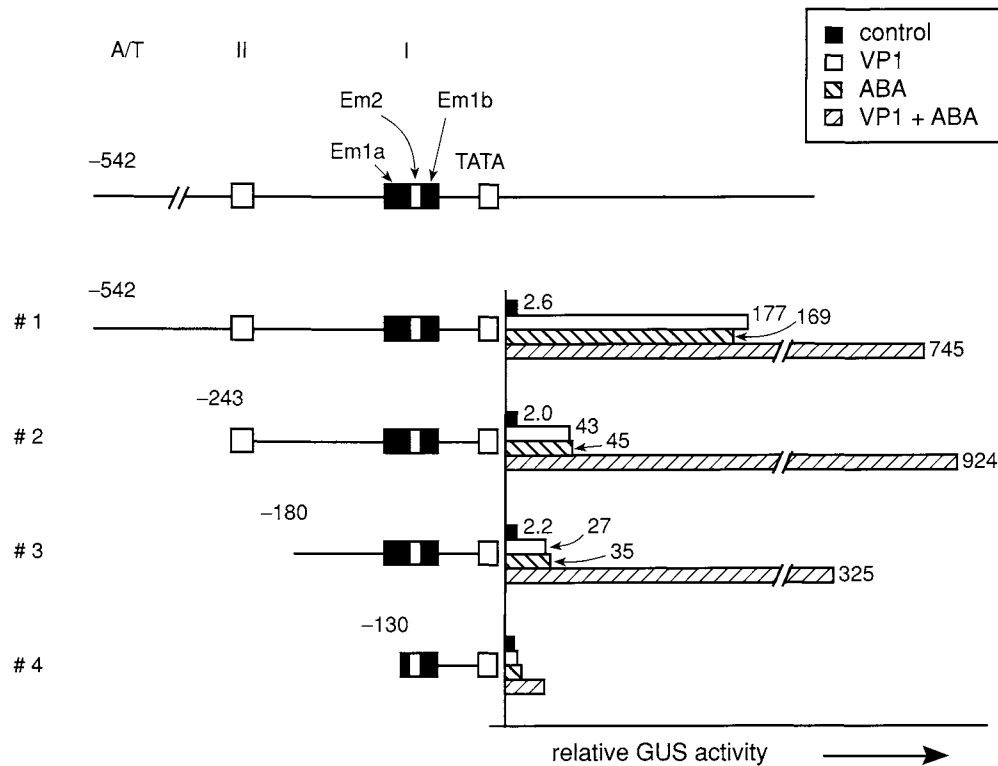


FIGURE 23-9 A schematic drawing of the EM promoter and localization of VP1 response sequence. The GUS reporter gene was fused to several deletion constructs of the EM promoter and cotransfected via electroporation, with or without a construct containing the VP1 coding sequence fused to a strong constitutive promoter (CaMV 35S) in maize protoplasts for transient assay. GUS expression was monitored \pm ABA (10^{-4} M). Construct #4 was disrupted in the EM1a element and caused a strong reduction in GUS activity. VP1 + ABA also have a strong synergistic effect on GUS activity. Drawn from data in Vasil *et al.* (1995).

of this domain or site-directed mutations in this domain lead to a loss in induction of genes. Hence, it is also suggested that TRAB1 recruits VP1 which activates transcription *via* its acidic domain. In an unrelated work, the B2 domain in VP1/AB13 has been

shown to be important for the expression of the Em and an albumin gene in seed tissues.

4.3.3.3. VP1 May Bind Directly to cis Sequence in Other Genes

The situation regarding C1 is different. VP1 is also specifically required for expression of the C1 gene, but the C1 promoter does not have an ACGT core-containing ABRE (see Table 23-1). Instead, it has two partially overlapping sequences, known as the Sph element, which have been shown to exclusively mediate its expression. The full sequence (5'-CGTGTCTCGTCCATGCAT-3') is required for induction by ABA, whereas the shorter sequence (in bold letters) is sufficient for induction by VP1. The transcription factor(s) binding to the Sph sequence is unknown, but induction by VP1 suggests that VP1 may interact directly with DNA. In support of such interaction, it has been shown that the B3 domain of the VP1 protein binds specifically to the Sph element *in vitro*. The B3 domain is found in a family of transcription factors seemingly involved in diverse functions [e.g., the DNA-binding domain of

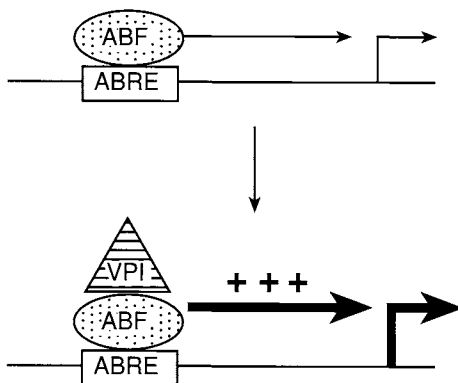


FIGURE 23-10 A model illustrating how VP1 could act as a coactivator of transcription through the ABRE. VP1 is shown binding to a transcription factor (ABF) through protein-protein interactions, while ABF binds to the ABRE. From Busk and Pagés (1997) with kind permission from Kluwer.

auxin-response factors (see Fig. 22-10, Chapter 22), and FUSCA3 of *Arabidopsis* responsible for accumulation of anthocyanin pigments].

4.3.3.4. VP1 Does Not Regulate All Seed-Specific Genes

Although VP1 or ABI3 regulates the expression of several seed-specific genes, they do not regulate all of them. For example, the *CAT1* gene in maize embryos, which encodes a catalase enzyme expressed in late embryogenesis, is not regulated by VP1. Similarly, it appears that VP1 discriminates between two response complexes. In barley, the response complex, consisting of an ABRE and the coupling element 3 (CE3) from the *HVA1* promoter, is activated by maize VP1 but not the ABRE with CE1 from the *HVA22* promoter (Fig. 23-11).

4.3.3.5. VP1 Protein Serves a Dual Regulatory Function

The VP1 protein serves a dual regulatory function: it specifically regulates gene expression during maize kernel development/maturation phase, while keeping the expression of germination-specific genes in check. As mentioned earlier, *vp1* mutation results in a premature expression of the α -amylase gene and softening of the endosperm. One of the mutant alleles of *vp1* carries a transposon insertion, which results in parts of a kernel showing *vp1* phenotype, while other parts of

the same kernel exhibit a wild phenotype. Thus, the kernel shows a mosaic endosperm with hydrolase activity localized to *vp1* mutant sectors (Fig. 23-12A). If the VP1 protein is expressed in these mutant sectors, it strongly represses the expression of a reporter gene coupled to a barley α -amylase promoter (Fig. 23-12B). In support, VP1 was shown to inhibit the GA-activated

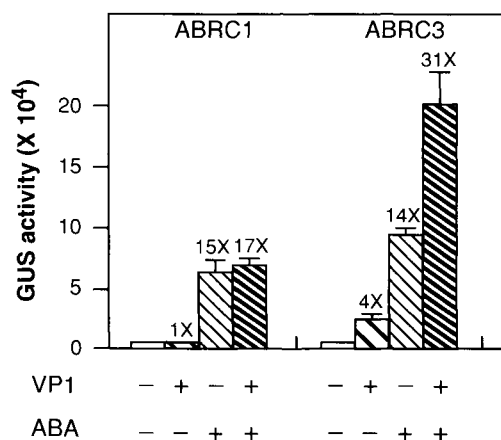


FIGURE 23-11 Activation of two response complexes by VP1. Response complexes, designated ABRC3 or ABRC1, consisted of an ABRE and coupling element 3 (CE3) from the *HVA1* promoter or an ABRE and the coupling element 1 (CE1) from the *HVA22* promoter in barley; they were fused to a minimal promoter and the coding sequence of a reporter gene. Each of the two constructs was cotransfected with the VP1-coding sequence driven by the 35S CaMV constitutive promoter into barley aleurone layers. Symbols below the bars indicate treatments with (+) or without (-) ABA or the VP1 effector construct. The relative GUS activity of each construct is the mean of four replicates. The error bar indicates the standard error of each set of replicates. X indicates fold induction. From Shen and Ho (1996).

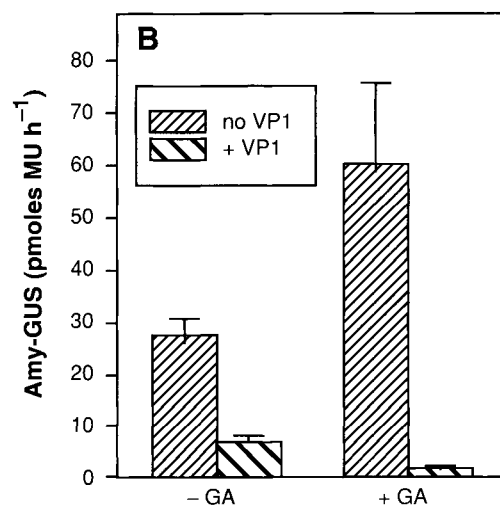


FIGURE 23-12 Maize kernel with mosaic endosperm and inhibition of α -amylase gene by VP1. (A) A mutant kernel carrying a *vp1-m2* allele, which is linked to a transposon and is expressed sporadically. The kernel, therefore, is a mosaic with purple patches (wild type) interspersed with colorless or yellow patches (these patches represent clonal sectors that have lost VP1 function). (B) Inhibition of *AMY-GUS* gene expression in aleurone tissue of *vp1 vp5* double mutant seeds (*vp5* is an ABA biosynthesis mutant; hence, the double mutant seeds lack ABA). Kernels were harvested 24 days after pollination and bombarded with constructs containing a barley α -amylase promoter fused to the reporter gene (*AMY::GUS* construct) \pm a construct consisting of the 35S CaMV promoter fused to *VP1*-coding sequence. Positively transfected kernels were exposed to \pm GA₃ (1.0 μ M) treatment, and expression of GUS was monitored. It can be seen that VP1 inhibited the expression of α -amylase as detected by GUS expression. Data represent mean of seven to eight replicates \pm SE. Reprinted with permission from Hoecker *et al.* (1996), © Cold Spring Harbor Laboratory Press.

α -amylase gene expression in barley aleurone independently of the action of ABA.

Thus, a key function of the *VP1* gene in maize is to ensure a proper integration of the maturation- and germination-specific programs in the seed. (Other signals, especially from the embryo, in control of these programs are not excluded.) An overexpression of *VP1* can cause ABA responsive genes to be expressed independently of ABA. It also appears that genes, or gene homologues, that are under the regulatory control of *VP1* during seed development/maturation cease to be susceptible to control by *VP1* when the seed enters the germinative program. Thus, certain isoforms of *C1* protein that are expressed during seed germination in maize are not subject to regulation by *VP1*.

4.4. Regulation of Expression of Stress-Related Genes

Chapter 16 showed that many genes induced by environmental factors, such as drought, salinity, or low temperature, are also induced by ABA. Further, evidence from ABA synthesis and response mutants indicates that the induction of these genes occurs by ABA-dependent and ABA-independent pathways.

An analysis of *RD29A* (also known as *COR78*, *LT178*) in *Arabidopsis*, which is induced by multiple stimuli, indicates two well-characterized *cis* elements in its promoter: an ABRE, which is responsive to ABA, and a 9-bp element (-TACCGACAT-) called DRE (dehydration responsive element), which is responsive to drought, salinity, or cold temperature, but not to ABA (see Table 23-1). Many cold-regulated genes have a 5-bp element (-CCGAC-) called the CRT or C repeat (or LTRE, low temperature regulatory element). As is evident, CRT is, in fact, part of the DRE sequence (shown in bold letters); hence, the DRE is also called DRE/CRT. The ABRE in the promoter is bound by a bZIP-type transcription factor, whereas the DRE is bound by a protein called DREB (for DRE-binding factor), and CRT is bound by a protein known as CBF (for C repeat-binding-factor) (Table 23-2). Thus, the gene can be regulated independently by drought, cold, or ABA.

CBF and DREB were thought to be encoded by small multigene families, but recent work shows that cDNAs of three members of the DREB family are identical to three members of the CBF family. Thus, DREB1A = CBF3; DREB1B = CBF1; and DREB1C = CBF2. These members of the DREB/CBF joint family are induced primarily by low temperature, not by osmotic stress or high salinity. A fourth member of the DREB/CBF family, DREB2A, in contrast, is regulated primarily by water stress and high salinity, not by low temperature. The two representative cDNAs, *DREB1A* and *DREB2A*,

TABLE 23-2 Regulation of Expression of *RD29A* Gene in *Arabidopsis*

Gene	<i>cis</i> element	Transcription factor
<i>RD29A</i> (<i>COR78</i> , <i>LT178</i>)	DRE/CRT ABRE	DREB or CBF bZIP

differ significantly in their deduced amino acid sequences, but nonetheless share a DNA-binding domain typical of EREBP/AP2-type transcription factors.

Induction of the *RD29A* gene in *Arabidopsis* plants exposed to water stress occurs in two phases. *RD29A* mRNAs begin to accumulate within minutes of onset of desiccation and then about 2–3 h later there is a larger rise. The initial rise is due to induction of the gene *via* the DRE/CRT element and the subsequent rise is mediated *via* the ABRE element and is correlated with a rise in the endogenous ABA content (Fig. 23-13). With the cloning of genes encoding DREB/CBF transcription factors, it has been shown that the transcripts of these proteins are expressed within minutes of exposure of plants to stress. Moreover, like most transcription factors, their overexpression in transgenic *Arabidopsis* evokes the expression of a whole battery of target genes without exposure of plants to stress conditions.

4.5. Summary of ABA-Induced Gene Regulation

Several functionally significant *cis* sequences have been identified in the promoters of ABA-regulated genes and in seed tissues, as well as in vegetative organs subjected to stress. One common *cis* sequence has an ACGT core, whereas others lack an ACGT core. Several transcription factors have also been identified. Many are bZIP proteins, which show a relaxed specificity for ACGT-containing ABREs and G box elements; others bind to Myc or Myb elements in promoters of stress-induced genes. Two seed-specific transcription factors, *VP1* in maize and *ABI3* in *Arabidopsis*, provide independent regulation of many seed-specific genes. Such regulation, at least in some cases, is mediated in concert with ABA-regulated *cis* sequences and transcription factors. The combinations of two or more *cis* sequences and the transcription factors that bind to them provide ABA-specific as well as organ specific expression to ABA-induced genes. While the response elements/complexes and transcription factors for several ABA-induced genes are known, links to further upstream elements of the ABA signaling cascade are unknown. Efforts are being directed to isolating mutants that are altered in ABA-induced gene expression using the

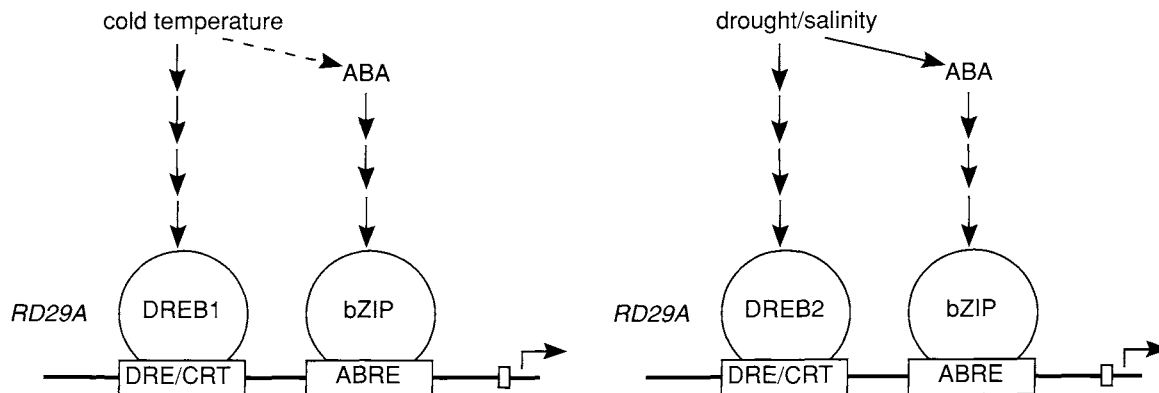


FIGURE 23-13 Models of signaling via DREB1A and DREB2A transcription factors. The induction of the *RD29A* gene in *Arabidopsis* plants exposed to low temperature occurs via DREB1A binding to DRE/CRT and a bZIP binding to the ABRE in the *RD29A* promoter. In plants exposed to water stress, the same gene is induced via DREB2A binding to the DRE/CRT element while a bZIP binds to the ABRE. ABA is indicated as being induced by low temperature or by water stress. Other parts of signaling are unknown.

technique of targeted genetics (for this technique, see Appendix 1). An *ade1* (ABA-deregulated gene expression) mutant was obtained, which shows altered expression of several genes when treated with ABA, but not by cold. An elucidation of these intermediates might also result from molecular genetic studies using ABA response mutants, which is the topic of the next section.

5. ABA RESPONSE MUTANTS

ABA response mutants are known from maize and mostly *Arabidopsis*. Some show insensitivity (or reduced sensitivity), whereas others show an enhanced sensitivity to ABA. Some are specific to tissues/organs, whereas others are more pleiotropic in their effects (see Table 23-3).

The *viviparous* (*vp*) mutants of maize were initially identified by vivipary, i.e., precocious germination (or sprouting) of kernel while still on the ear. Many *vp* mutants are ABA synthesis mutants (see Fig. 10-8 Chapter 10), but *vp1* is a response mutant and, as explained in an earlier section, the wild-type gene encodes a seed-specific transcription factor. ABA-insensitive mutants in *Arabidopsis* were isolated by utilizing the well-known phenomenon of inhibition of seed germination by moderate to high concentrations of ABA (see Chapter 19). The screen, therefore, was to select for seeds capable of germinating in the presence of ABA concentrations (3–10 μM) that were inhibitory to the wild type. Mutations at five loci, *ABI1*–*ABI5* (*ABSCISIC ACID INSENSITIVE*), have been identified; all mutants are ABA insensitive and, as explained earlier, the wild-type gene *ABI3* encodes a seed-specific transcription factor that is homologous

to VP1. A screen for lack of seed germination under ABA concentrations that are too low (0.3 μM) to inhibit the germination of wild-type seeds has yielded mutations at two loci. These loci are called *ERA1* and *ERA2* (*ENHANCED RESPONSE to ABA*). The *ERA1* locus has been investigated in greater detail.

In addition to the mutants listed in Table 23-3, there are other insensitive mutants, e.g., *cool* in barley, which shows insensitivity in guard cells, *rea* (*red embryonic axis*) in maize, and *gca1/8* (growth control by ABA) in *Arabidopsis*. These mutants are not considered in this chapter.

5.1. ABA-Insensitive Mutants

The *abi4* and *abi5* mutants are thought to act in the same pathway as *abi3*. The *ABI4* and *ABI5* genes have been cloned; they encode a transcription factor with an AP2-type DNA-binding domain and a b-ZIP type transcription factor, respectively, but their precise roles are still being worked out. The *abi1-1* and *abi2-1* mutants are both semidominant mutations at different loci. (Some additional alleles at the two loci have been identified recently.) The two mutants are phenotypically similar and pleiotropic in their ABA insensitivity. As illustrated in Fig. 23-14, they require much higher ABA concentrations to inhibit seed germination or inhibit root growth than the wild type; stomatal pore closing does not occur to the same extent as in the wild type, resulting in much greater water loss from leaves under conditions of water stress, and ABA-induced gene expression is much reduced. In addition, maturing seeds of the mutants show reduced dormancy. All these results indicate that the mutants are much less sensitive to applied ABA than the wild type. The endogenous ABA concentrations in these

TABLE 23-3 Main Characteristics of Some Mutations Known to Affect ABA Sensitivity^a

Species	Mutation (dominance ^b)	Phenotypic effect	Gene product ^c
Insensitive mutants			
<i>Zea mays</i>	<i>vp1</i> (r)	Restricted to seeds	Transcription factor
<i>Arabidopsis thaliana</i>	<i>abi1</i> (sd)	Pleiotropic	Protein phosphatase 2C
	<i>abi2</i> (sd)	Pleiotropic	Protein phosphatase 2C
	<i>abi3</i> (r)	Restricted to seeds	Transcription factor
	<i>abi4</i> (r)	Probably seeds	Transcription factor (AP2 type)
	<i>abi5</i> (r)	Mostly seeds, also vegetative tissues	Transcription factor (bZIP type)
Hypersensitive mutants			
<i>A. thaliana</i>	<i>era1</i> (r)	Pleiotropic	β subunit of farnesyl transferase
	<i>era2</i> (r)	Pleiotropic	Not known

^aFrom data in Leung and Giraudat (1998) and Finkelstein and Lynch (2000).^bDominance of mutant alleles over wild type: d, dominant; sd, semidominant, and r, recessive.^cFunction of the product of the wild-type gene.

mutants are nonetheless comparable to those in the wild-type plants.

While the two mutations are similar in many respects, they also show some differences. For example, the *abi1* mutants show an enhanced induction of lateral roots in the presence of inhibitory concentration of ABA, whereas the *abi2* mutants do not show this feature.

5.1.1. ABI1 and ABI2 Genes Encode Protein Phosphatases

ABI1 and *ABI2* are homologous genes and encode protein phosphatases of type 2C (PP2C) (Fig. 23-15). They are unusual phosphatases. They have the typical Ser/Thr type catalytic domain near the C terminus (the two mutations result from a single amino acid change, from Gly¹⁸⁰ to Asp in *abi1* and from Gly¹⁶⁸ to Asp in *abi2*, both in the catalytic domain), but their amino-terminal has no significant homology to any available protein sequences in the data bases.

5.1.2. Function of ABI1 and ABI2 Proteins

Both genes are induced by exogenous ABA; they are also induced when plants are subjected to osmotic stress, which raises endogenous ABA levels.

ABI1 and *ABI2* proteins expressed in bacterial cells function as phosphatases *in vitro*. They probably serve

as phosphatases *in vivo* also, but their substrates *in planta* and the precise steps at which they act in ABA signal transduction pathway are mostly unknown. Because their mutant phenotypes are pleiotropic in effects, they probably act at some early step in ABA signal transduction. For instance, for ABA-induced stomatal pore closure, it has been shown that in *abi1-1* and *abi2-1* mutants, the ABA-induced rise in cytosolic Ca²⁺ is reduced compared to the wild type such that the slow anion channels are unable to open. However, if exogenous Ca²⁺ is supplied to the guard cells, the slow anion channels are able to open. Thus, it appears that the lesion in these mutants is in some step between ABA perception and the rise in cytosolic Ca²⁺ that requires dephosphorylation. The overlapping, but nonetheless distinct, effects of *abi1-1* and *abi2-1* mutations suggest that *ABI1* and *ABI2* perform similar functions, but possibly in different pathways. The N-terminals of the two proteins are dissimilar, which may indicate that their activities are regulated by different regulatory proteins. The regulatory proteins may, in turn, dictate substrate specificities and cellular locations of these phosphatases.

As mentioned earlier, *abi1-1* is a dominant mutant allele of *ABI1*. Hence it was not possible to decide how *ABI1* regulates ABA signaling. Jérôme Giraudat at the Centre National de la Recherche Scientifique, Gif-

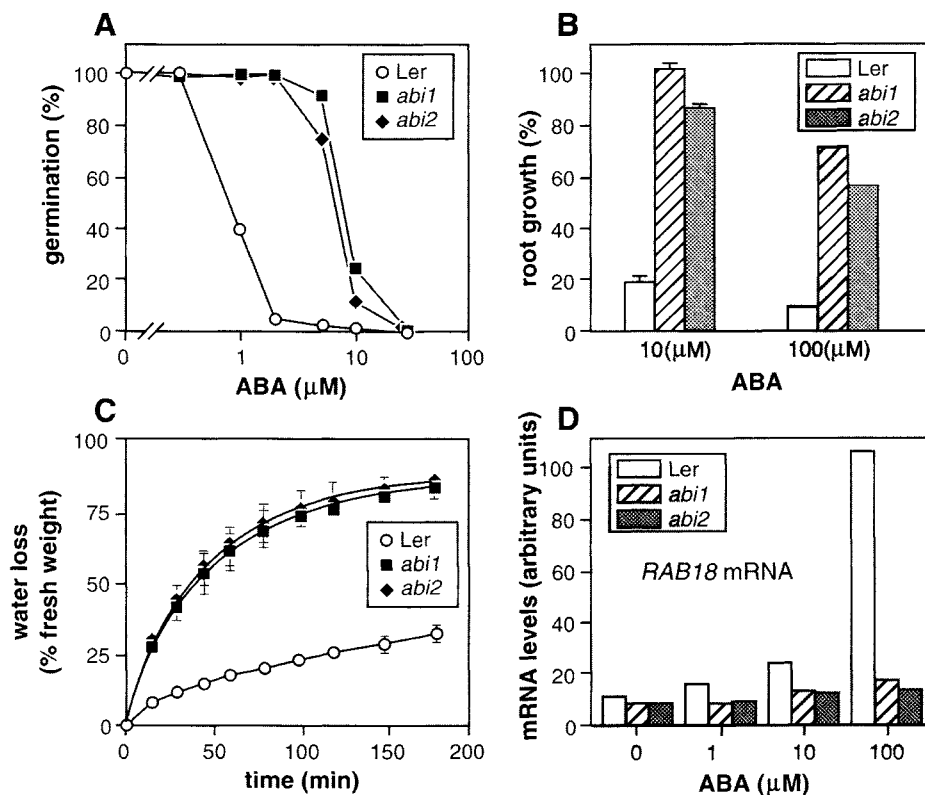


FIGURE 23-14 Pleiotropic effects of *abi1* and *abi2* mutations. Responses of *abi1-1* and *abi2-1* mutants in homozygous state and of wild-type *Arabidopsis* (*Landsberg erecta*, *Ler*) to exogenous application of ABA or to wilting. (A) Inhibition of seed germination by ABA. Germination is defined as the emergence of the radicle tip. Seeds were chilled for 3 days at 4°C in darkness and incubated for 3 days at 21°C with a 16-h light photoperiod. The number of germinated seeds is expressed as the percentage of the total number of seeds plated (40–80). (B) Inhibition of root growth. Seeds were germinated for 5 days on ABA-free medium. Seedlings were then transferred to media with ABA concentrations as shown and root length was scored after 4 days. Root growth of ABA-treated seedlings is expressed as percentage relative to control in ABA-free medium. Values are means \pm SE from samples of 11–15 seedlings each. (C) Kinetics of water loss from excised leaves. Three to four leaves were excised from each plant, and their total fresh weight was measured at different times while they were allowed to wilt under laboratory conditions. Water loss is expressed as the percentage of initial fresh weight. Values shown are mean \pm SD of measurements with five individual plants per genotype. (D) ABA induction of *RAB18* transcript. Total RNA was extracted from plantlets grown for 5 days on ABA-free medium and then transferred to media containing the indicated concentrations of ABA for 55 h. RNA blots were hybridized with the *RAB18* probe and normalized with an independent probe. *RAB18* mRNA levels are expressed in arbitrary units, with 1 unit corresponding to the mRNA level in wild-type (*Ler*) plantlets on ABA-free medium. From Leung *et al.* (1997).

sur-Yvette, France, and colleagues created loss-of-function mutations by mutagenizing *abi1* seeds and

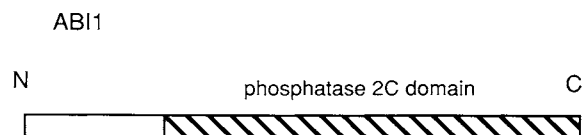


FIGURE 23-15 Structure of ABI1 protein in *Arabidopsis*. The protein (434 amino acids) has an amino-terminal domain and a carboxy-terminal domain homologous to the 2C class of serine-threonine protein phosphatases.

screening for intragenic suppressors of the *abi1* phenotype. The revertant mutants were selected on the basis of their similarity to the wild type in ABA-mediated responses. All were found to be mutations in the catalytic domain of ABI1 and lacked a detectable phosphatase activity in *in vitro* assays. Since they were loss-of-function mutations, it could be concluded that ABI1 phosphatase acts as a negative regulator of ABA responses. The exact manner in which the ABI1 phosphatase attenuates the ABA signal is unknown, but most likely it does so by dephosphorylating a key protein in the signal transduction chain. By hom-

ology, ABI2 is thought to act similarly as a negative regulator.

5.1.3. Phosphorylation/Dephosphorylation Is an Essential Component of ABA Signaling

Many other studies provide circumstantial evidence of protein phosphorylations and dephosphorylations playing a role in ABA signaling. These studies have relied on a demonstration that protein kinases (PKs) or protein phosphatases (PPs) are induced or activated as a result of ABA treatment. For instance, *RD29B* gene in *Arabidopsis* is induced by ABA. It has two ABREs in its promoter, which are transcriptionally activated by binding of two bZIP transcription factors, AREB1 and AREB2. A specific ABA-activated 42 kDa protein kinase is required to phosphorylate the N terminal regions of AREB proteins. Pharmacological experiments utilizing inhibitors of PKs or PPs have also been used to demonstrate an effect on either stomatal behavior or ABA-induced gene expression. Most of these studies are in preliminary stages and are difficult to evaluate because either the identity of the kinases or phosphatases are unknown or their substrates are unknown.

5.2. Mutants with Enhanced Sensitivity to ABA

As mentioned earlier, mutations at *ERA* loci give enhanced sensitivity to ABA, i.e., the seeds do not germinate at concentrations of ABA that are too low to inhibit the germination of wild-type seeds. Enhanced sensitivity to ABA also means enhanced dormancy in relation to wild type. This is shown for *era1-1* seeds, which, given identical chilling treatments, take longer to germinate than wild-type seeds (Fig. 23-16).

The *ERA1* gene encodes the β subunit of a farnesyl transferase (FTase) in *Arabidopsis*. FTases are prenyl transferases that catalyze the transfer of farnesyl units (from farnesyl diphosphate) to proteins. They are heterodimers with a regulatory subunit α and a catalytic subunit β . Farnesylation (or lipidation) of proteins facilitates their integration or anchoring in membranes and their cellular functioning. Several proteins involved in signal transduction in yeast and mammalian cells, such as the α and γ subunits of heterotrimeric G proteins and small GTP-binding proteins of the Ras family (see Chapter 25), are so farnesylated.

The function of *ERA1* in ABA signaling is not known, but it has been shown that *era1-2*, a null mutant in which the coding sequence of *ERA1* is de-

leted and which has no FTase activity, is affected in various developmental phenomena, including cellular differentiation in vegetative and floral meristems, meristem maintenance, and regulation of flower development. *era1-2* mutants also show an enhancement in the ABA-induced anion current and much greater pore closing than wild-type plants. In a graphic illustration of the importance of *ERA1* FTase in stomatal behavior, it was shown that, under water stress, *era1-2* mutants were able to stay green and turgid and survive long periods of drought, whereas the wild-type plants showed severe chlorosis and died (Fig. 23-17).

Since mutant alleles at the *ERA1* locus are recessive and cause hypersensitivity to ABA, it may be concluded that *ERA1* is a negative regulator of ABA-induced stomatal closure. However, *ERA1* FTase seems to have many more wide-ranging effects on plant development, and enhanced sensitivity to ABA may be part of a much broader role that FTases, or prenylation in general, play in plant development and response to stress.

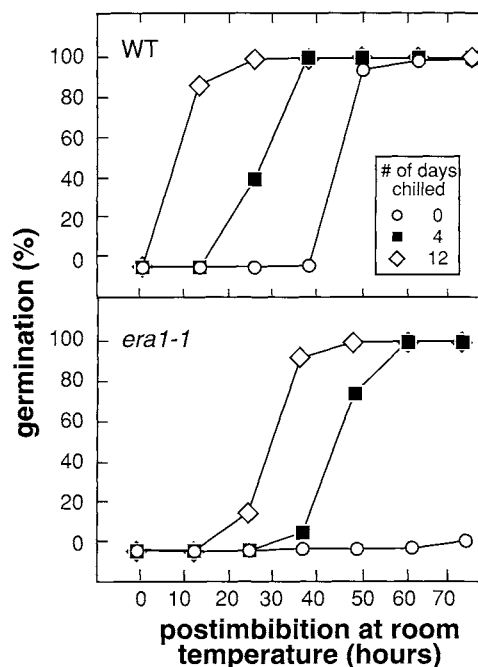


FIGURE 23-16 Germination of wild type (WT, Columbia) and *era1-1* mutants of *Arabidopsis*. Mature seeds were chilled for 0, 4, and 12 days at 4°C in darkness before being plated for germination at room temperature. Germination was scored as positive when a radicle tip had protruded. Each experiment was performed in triplicate and each point represents a germination test of 40–50 seeds. The percentage of germination was determined by dividing the number of seeds that germinated at a given time by the total number of seeds plated. Modified with permission from Cutler *et al.* (1996), © American Association for the Advancement of Science.

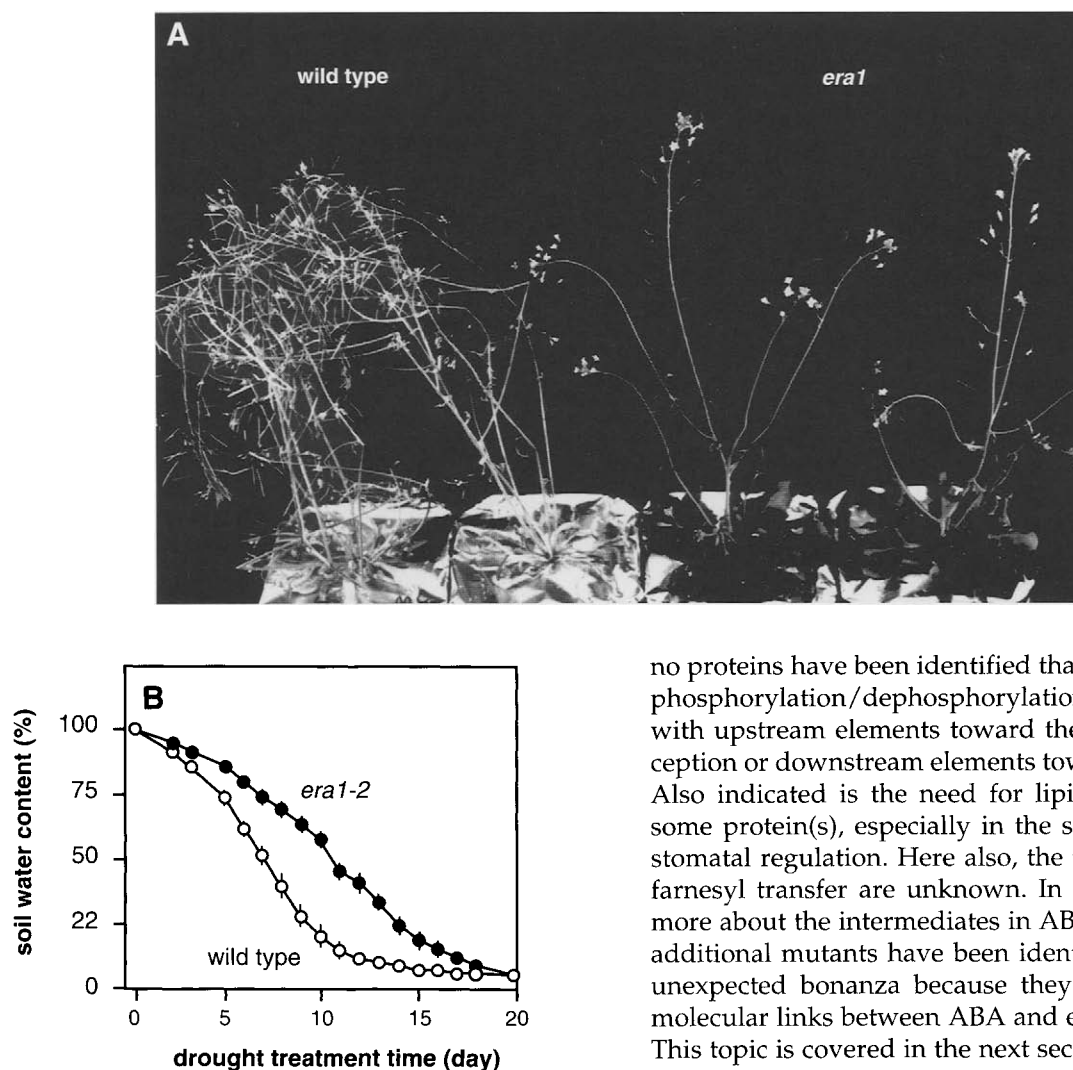


FIGURE 23-17 Reduced wilting of *era1-2* plants during drought stress. (A) Both *era1-2* and wild type plants were grown under normal watering conditions for ~ 21 days and were then subjected to water stress by terminating irrigation. Pots were covered to minimize evaporation from soil. Photograph was taken after 12 days of drought stress. *era1-2* plants were able to survive because their stomatal pores were closed to a larger extent than in the wild-type plants. (B) Changes in soil water content during drought stress treatment of wild type and *era1-2* plants. The higher soil water content in pots holding *era1-2* plants (than in pots holding wild type plants) indicates lesser water loss by transpiration. Reprinted with permission from Pei *et al.* (1998), © American Association for the Advancement of Science.

5.3. Section Summary

The response mutants isolated of far indicate clearly that ABA signaling involves a change in the phosphorylation status of a protein (or proteins) in the signal transduction chain. Such a change in the phosphorylation status is required both for ABA-induced stomatal pore closing and for gene transcription, but so far

no proteins have been identified that are the targets of phosphorylation/dephosphorylation or that interact with upstream elements toward the site of ABA perception or downstream elements toward ion channels. Also indicated is the need for lipid modification of some protein(s), especially in the signaling chain for stomatal regulation. Here also, the target proteins for farnesyl transfer are unknown. In an effort to know more about the intermediates in ABA signaling, some additional mutants have been identified, which is an unexpected bonanza because they provide the first molecular links between ABA and ethylene signaling. This topic is covered in the next section.

6. CROSS TALK BETWEEN ABA AND ETHYLENE SIGNALING

Physiological data show that many plant responses are mediated by more than one hormone. Redundancy is one of the hallmarks of plant signaling. For instance, root growth is inhibited by auxins above a certain concentration; it is also inhibited by brassinosteroids, ABA, ethylene and jasmonates. Seed germination is inhibited by ABA and is promoted by gibberellins (GA) and ethylene. In contrast, there are some responses that are exclusively mediated by one or another hormone. For instance, genes induced in desiccation tolerance seem to be induced exclusively by ABA and, of course, desiccation. Evidence has accumulated in recent years that provides the molecular basis for cross talk between ABA and ethylene signaling and the possible site for such interaction.

Crucial evidence comes from mutants isolated in screens for sensitivity to ABA inhibition of seed germination. Homozygous *abi1* seeds were mutagenized and screened for enhancement or suppression of the *abi1* phenotype. What came as a remarkable double surprise was that one of the enhancer mutants mapped to the same locus as *CTR1* in ethylene signaling (see Chapter 21). Sequencing data confirmed that it was a mutated allele of *CTR1*; it was renamed *abi1/ctr1-10*. In contrast, the suppressor screen yielded a mutant that was mapped to the *EIN2* locus in ethylene signaling and confirmed to be a mutated allele at that locus; it was renamed *abi1/ein2-45*. However, the surprises did not end there. In the original screen for *era* mutants, three loci were identified. *ERA1*, as mentioned earlier, encodes the β subunit of a farnesyl transferase. Not much is known about *ERA2*, but the *ERA3* locus has been found to be identical to *EIN2*, and *era3-1* and *era3-3* mutant alleles have been renamed *ein2-44* and *ein2-46*. (*era3-2* is the same as *abi1/ein2-45*.)

This remarkable chain of events puts fresh light on ABA signaling and the role of proteins known to mediate ethylene signaling. The newly discovered mutated alleles are recessive, as are the hitherto known mutant alleles of *CTR1* or *EIN2*. Let us look at some data on effects of *ein2* and *ctr1* mutations on some of the key responses mediated by ABA. For comparison, data from *etr1*, a receptor mutant in ethylene signaling, are included.

etr1-1 and *ein2-45* (also *ein2-44* and *ein2-46*) mutations all enhance the sensitivity of seeds to ABA inhibition of germination; i.e., seeds are inhibited from germination at lower concentrations of exogenous ABA than the *abi1* mutant and the wild-type seeds (Fig. 23-18). In contrast, the *ctr1-10* mutation reduces the sensitivity to ABA: mutant seeds require much higher concentrations of ABA to get 50% inhibition of germination. Since a lessened sensitivity to ABA inhibition of seed germination goes hand in hand with enhanced seed dormancy (because the sensitivity to ABA is affected), seeds are more dormant in *etr1/ein2-45* mutants than in the wild type.

Data just given indicate that several key proteins in ethylene signaling, *ETR1*, *CTR1*, and *EIN2*, impinge on ABA signaling in seeds. Among these, *EIN2* is especially significant—it seems to be at the crossroads of multiple hormone response pathways and its structure seems to be conducive to such multiple signaling (see Chapter 21). Since the known mutations in *EIN2* are recessive and increase the sensitivity to ABA, *EIN2* is a negative regulator of ABA signaling in seeds, while being a positive regulator of ethylene signaling. Also, as discussed in Chapter 19, seed germination and dormancy are affected by GAs. Gibberellin signaling and

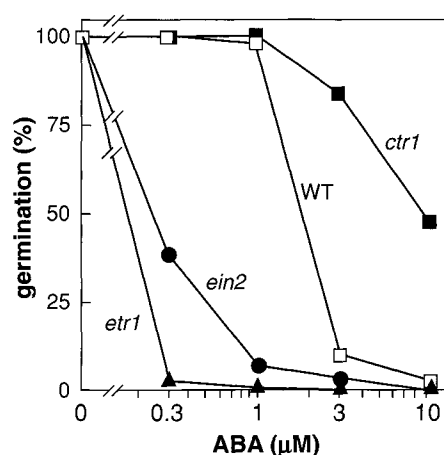


FIGURE 23-18 ABA dose response for inhibition of germination. Seeds were plated on medium supplemented with the indicated concentrations of ABA, chilled for 4 days at 4°C in darkness, and incubated at 21°C with a 16-h light photoperiod. The number of fully germinated seeds (with fully emerged radicle tip) is expressed as the percentage of the total number of seeds plated (100–200). Wild-type seeds are from the Columbia ecotype. From Beaudoin *et al.* (2000).

GA/ABA interaction are dealt with in the next chapter, but a model for seed germination as affected by the three hormones, ABA, ethylene, and GAs, is presented in Fig. 23-19.

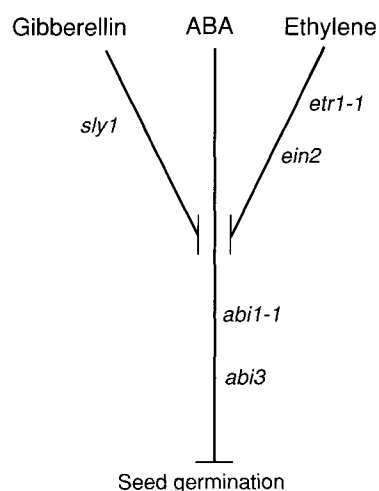


FIGURE 23-19 Schematic representations of the interactions among ABA, ethylene, and GA signaling in the regulation of seed germination. Ethylene and GA signaling cascades promote seed germination by decreasing sensitivity to endogenous ABA in imbibed seeds. The *sly1* mutant is GA insensitive, and the *etr1-1* and *ein2* mutants are ethylene insensitive—in those backgrounds, sensitivity to ABA is enhanced. The *abi1-1* and *abi3* mutants are ABA insensitive—seeds of these mutants require higher concentrations of ABA than wild type seeds to inhibit germination. Positions of the interactions among ABA, ethylene, and GA pathways are speculative. From Beaudoin *et al.* (2000).

In contrast to seed behavior, root growth in *ein2-45* (also *etr1-1*) displays a markedly reduced sensitivity to inhibition by ABA. As expected from their ethylene insensitivity, these mutants are also resistant to the ethylene-induced inhibition of root growth, but *abi1-1* is not. However, ABA inhibition of root growth is not mediated by an increase in ethylene synthesis.

While the ABA-induced seed and root responses are affected in *ein2-45* or *ctr1-10* mutants, some other ABA responses, such as stomatal behavior and induction of *RD29A* or *RAB18* genes, are not affected. This agrees with the known lack of ethylene effects on the regulation of stomatal behavior and the induction of desiccation tolerance.

7. CHAPTER SUMMARY AND CONCLUSIONS

Research since the mid-1980s has clarified many aspects of ABA-induced gene expression and the interaction of ABA and environmental factors, such as drought, salinity, and cold temperature, in the induction of genes. Many *cis* elements have been defined, and some transcription factors have been identified and cloned. VP1 and ABI3 are seed-specific transcription activators that are responsible for the induction, and repression, of genes at a specific stage in plant development, i.e., during seed development and maturation. They exercise this control by regulating the activities of other transcription factors. This is among the first examples of organ-specific control in plant gene expression. Some ABA-insensitive mutants have been identified whose wild-type genes encode protein phosphatases, ABI1 and ABI2. Because these mutants are pleiotropic in their effects, it is reasonable to assume that ABI1 and ABI2 phosphatases are involved in some early step in ABA signaling, but their target proteins are unknown. Some mutants hypersensitive to ABA have also been identified; and the gene for one of them, *ERA1*, encodes the β subunit of FTase, an enzyme known to add lipid moieties to proteins prior to their association with membranes. The role played by ERA1 FTase in ABA-mediated responses may be part of a broader role that lipid modifications of proteins play in plant development and morphogenesis and in stress responses. ABA-induced stomatal pore closure is mediated via an increase in free cytosolic Ca^{2+} , which, in turn, leads to an activation of the slow anion channels and depolarization of the plasma membrane. Depolarization results in opening of the K^+ efflux channel and exodus of K^+ (and other solutes) from the guard cell, leading to loss of turgor and closure of the pore. It also seems that ABI1

and ABI2 phosphatases are involved at some step between a rise in the cytosolic Ca^{2+} and opening of the slow anion channels. However, the particulars of ABA signaling and targets of ABI1 and ABI2 phosphatases and FTase in stomatal regulation are not known. One of the biggest challenges is that no single protein has been found as yet that can be considered an ABA receptor. Moreover, the site of ABA perception, whether extracellular or intracellular, is also unclear. Many of the intermediate steps between signal perception and activation of transcription factors, or regulation of ion channels in guard cells, are also unclear. Meanwhile, exciting cross links between ABA and ethylene signaling have been found.

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Molecular Biology of Action of Gibberellins, Cytokinins, Brassinosteroids, and Jasmonates

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Chapters 21–23 presented current information on the molecular aspects of signaling by ethylene, auxins, and abscisic acid (ABA). These three hormones were singled out for separate treatment because the information on each is unique in some ways. Signaling by a plant hormone is better and more completely known for ethylene than that for any other hormone. Hormone-binding proteins are better characterized for auxin than for other hormones, and more is known about ABA-regulated gene expression than for other hormones. In comparison, our information on the remaining hormones, gibberellins (GAs), cytokinins (CKs), brassinosteroids (BRs), and jasmonates (JAs),

is still fragmentary. This chapter summarizes that information.

SECTION I. MOLECULAR BIOLOGY OF GIBBERELLIN (GA) ACTION

1. GA RESPONSES

As explained in earlier chapters, gibberellins play major roles in the elongation growth of stems, petioles, and leaves; the flowering response; seed germination; and mobilization of reserve foods in grass seeds. They also act as general stimulators of fruit growth, probably by creating a sink for photoassimilate, and in expression of the male sex in flowers. This chapter first considers GA-binding proteins and the GA site of perception; then the *cis* sequences and transcription factors involved in GA-induced gene expression; and finally GA response mutants and how GA signaling might be affected.

2. GA-BINDING PROTEINS

Soluble proteins from elongating tissues of several plants (pea epicotyls, cucumber and Azuki bean hypocotyls, maize and rice leaves) have been shown to bind radiolabeled GA₁ or GA₄ with high affinity and specificity. These assays have utilized both *in vivo* and *in vitro*-binding assays. In general, there is good agreement between the biological activities of various GAs and GA derivatives and their abilities to displace radiolabeled GAs from binding sites. The binding sites have been shown to be proteins, and the optimal pH for binding is about 7.0–7.5. The calculated K_D values range from about 700 pM to 140 nM, a range that agrees with the GA concentrations required for elongation growth in these systems. The proteins have not been purified, however, and it is unclear whether binding is to a GA receptor or to one of the enzymes in GA metabolism.

Other studies using photoaffinity-labeled GA₄ have identified binding proteins in plasma membrane fractions from oat aleurone protoplasts, as well as pea and *Arabidopsis* stem tissue. The binding was competed for by biologically active GAs, but not inactive GAs. The identity of proteins is still unknown, but one 18-kDa protein was partly sequenced, which may lead to its identification.

3. SITE OF GA PERCEPTION

While there is considerable evidence for GA-binding activity in soluble fractions from a number of plants, there is also increasing evidence that GA perception occurs at the outer surface of the plasma membrane. Richard Hooley and associates at Long Ashton Research Station, University of Bristol, have synthesized gibberellins that are impermeable across membranes. For instance, GA₄ was derivatized at C-17 and attached *via* a hydrophobic spacer arm to 120- μ m-diameter Sepharose beads such that only the unmodified end, which included the biologically active A and B rings of the GA molecule, could permeate the plasma membrane to a depth of about 1.95 nm (Fig. 24-1).

Such derivatized GA₄ could still elicit expression of α -amylase genes in isolated aleurone protoplasts, albeit to a much less extent than the parent, underivatized GA₄ (Table 24-1). However, the intact aleurone layers, with cell walls, could not be reached by the derivatized GA₄ and, as expected, gave little response.

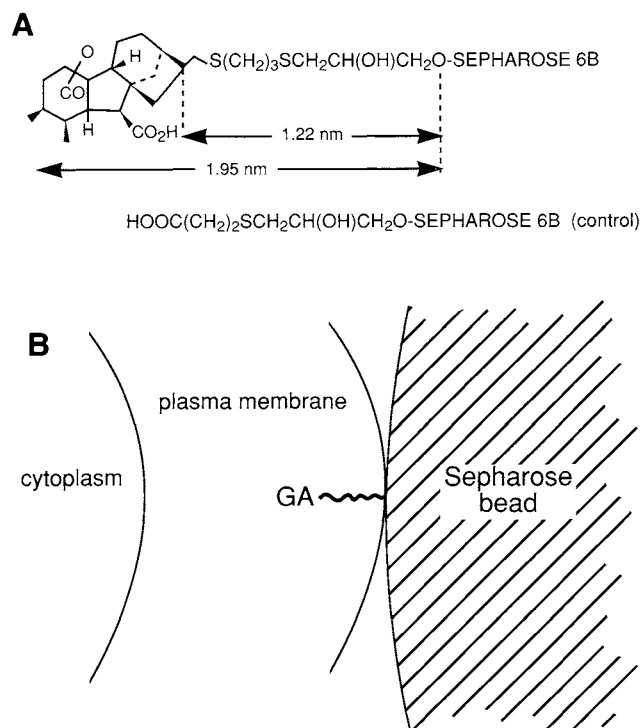


FIGURE 24-1 Derivatized GA₄ and its penetration into the plasma membrane of oat aleurone protoplasts. (A) Structure of the 17-thiol derivative of GA₄ (top row) and control Sepharose 6B (bottom row). (B) A model of plasma membrane and derivatized GA₄ linked to a Sepharose bead (not drawn to scale). The derivatized GA molecule cannot enter the plasma membrane except to a depth of about 2.0 nm. Adapted from Hooley *et al.* (1991).

TABLE 24-1 α -Amylase Production by Underivatized GA₄ and Derivatized GA₄ in Oat (*Avena fatua*) Aleurone Protoplasts^a

GA concentration (M)	Total α -amylase (units) ^b	
	GA ₄	GA ₄ -17-S-(CH ₂) ₃ -SH
1.0×10^{-5}	—	48.1
1.0×10^{-6}	51.2	44.3
1.0×10^{-7}	53.6	31.3
1.0×10^{-8}	43.1	16.7
1.0×10^{-9}	30.7	2.8

^aFrom Hooley *et al.* (1991).

^bMinus GA control (1.07 Units).

Similar experiments with GA₄ 17-sulfonic acid, which has a pK_a of 0.6 and therefore occurs mostly as an impermeable anion, gave identical results.

In other studies, Russell Jones and associates at University of California, Berkeley, used barley aleurone protoplasts, which were immobilized by embedding in a thin film of agarose. Microinjection of GA₃ into the protoplasts elicited none of the three GA-induced responses, i.e., increased vacuolation, secretion of α -amylase, and expression of a reporter gene under the control of a barley α -amylase promoter. The addition of GA₃ to the external medium, however, stimulated all three responses.

Additional evidence that GA is perceived at the cell surface comes from studies involving transmembrane receptors, albeit of two different kinds. As explained in Chapter 25, one class of plasma membrane-based receptors works in concert with heterotrimeric GTP-binding proteins. The receptor, on perceiving the signal, undergoes a conformational change, which is sensed by the neighboring heterotrimeric G-protein complex, which in turn hydrolyzes GTP to GDP and starts a signaling cascade. The cascade has multiple effects, which range from opening or closing of ion channels to gene expression. Some agonists, such as the peptide Mastoparan 7 (Mas7), can trigger the G-protein cascade in the absence of an external signal. Using oat aleurone protoplasts and layers, it was shown that supplying Mas7, in the absence of any exogenous GA, is sufficient to trigger the expression of the reporter gene, *GUS*, under the control of an α -amylase promoter (Fig. 24-2).

A *dwarf1* mutant of rice is GA insensitive in both α -amylase production by embryoless half-seeds and stem elongation of seedlings. The *Dwarf1* gene encodes the α subunit of a heterotrimeric G protein, which supports the concept that GA is perceived by a plasma

membrane-based receptor and involves signaling via a G protein.

The second type of plasma membrane-based receptors are transmembrane receptor-like kinases. Deepwater rice shows enormous elongation of young internodes in response to flooding, and the response has been shown to be due to GA (see Chapter 15). A gene encoding one of the receptor-like kinases (*OsTMK1* for *Oryza sativa* trans-membrane kinase) has been isolated. The expression of the gene is upregulated by GA in zones of cell division and cell elongation.

In summary, studies on aleurone tissue, *dwarf1* rice, and deepwater rice present strong arguments for an extracellular site for GA perception. However, GA-

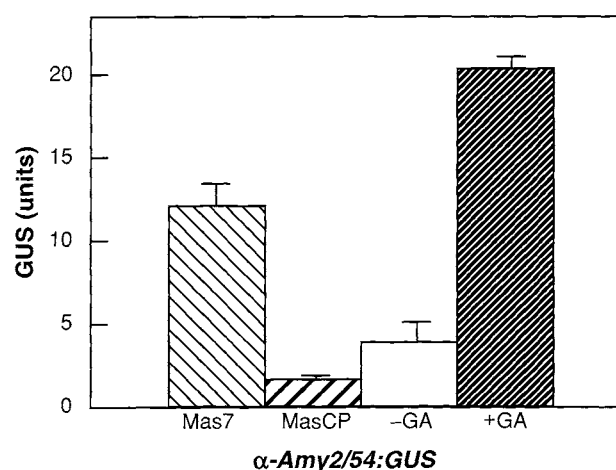


FIGURE 24-2 Expression of α -Amy2/54::GUS construct in oat aleurone protoplasts. Freshly prepared aleurone protoplasts were transformed by the construct and incubated for 5 days with Mastoparan 7 (Mas7), its inactive analog (MasCP), both 3 μ M or GA₁ (50 nM), or without GA₁. Mas7 could induce the expression of GUS in the absence of exogenous GA₁. From Jones *et al.* (1998).

binding studies using GA responsive stem or leaf tissues favor an intracellular site for GA perception. While it is possible that gibberellins are perceived differently in different systems, the balance favors a surface perception of GA. The issue cannot be resolved, however, without the identification and isolation of the GA receptor(s).

4. GA-INDUCED GENE EXPRESSION

Gibberellin-induced stem and leaf elongation, flower development, seed germination, and mobilization of seed food reserves all involve gene expression. Among these, the best studied is gene expression in the aleurone tissue of cereal grains. GA-induced production and secretion of α -amylases and other hydrolases in cereal grains were explained in Chapter 19. This section deals with the regulation of gene expression in the aleurone system, followed by gene expression in two other systems: stem elongation and flower development.

4.1. Aleurone tissue

Among the various genes upregulated by gibberellins in the aleurone system, α -amylase genes in barley, wheat, and rice are the best characterized, and this account pertains specifically to them. The α -amylase gene family is divided into three subgroups based on amino acid homology and gene structure (i.e., the number and placement of introns). In wheat and barley, two subgroups are expressed in aleurone tissue during seed germination; the high *pI* (α -Amy1) and low *pI* (α -Amy2) groups. Both are induced by GA, but their temporal pattern of expression differs. In rice, there is only a single α -Amy2 representative, and it is expressed at a very low level. Sequence comparisons in the proximal promoters of genes in both high *pI* and low *pI* subgroups in wheat and barley show

considerable homologies within each subgroup, but not between the two subgroups. The third α -amylase subgroup, α -Amy3, is expressed in wheat only during seed development, not during germination. In rice, however, several α -Amy3 genes are expressed in aleurone cells and in the scutellum during germination.

4.1.1. *cis* Elements in α -Amylase Genes

Studies using deletion constructs from promoters of α -amylase genes in all three subgroups have shown that the proximal promoter, to -300 bp from the transcription initiation site, is important for GA-regulated gene expression. Within this region, sequence comparisons among high *pI* and low *pI* genes of wheat and barley have led to an identification of three conserved sequences, including the TAACAA/GA core element, which is the GA response element (GARE) (the sequences are numbered 1–3, Fig. 24-3). Mutations in this core element cause severe disruption of reporter gene expression in transient assays. In low *pI* or α -Amy2 genes in barley, wheat, and oat, functionally important *cis* elements also occur upstream from the three boxes mentioned earlier (see Fig. 24-3). These include the Opaque-2 element, which forms part of box 2. (The Opaque 2 element, in combination with an ACGT core-containing ABRE, can give ABA-specific expression.)

The relative placement or order and orientation of these sequences, although not the spacing between them, are conserved. Functional analysis using aleurone layers (or protoplasts) and transient GUS expression reveals that while the GARE is essential for GA₃-induced α -amylase expression in both high *pI* and low *pI* α -amylase promoters, it works in concert with other elements. For the high *pI* α -amylase genes in barley, the GARE, together with the most proximal element, the TATCCAC/T box, is sufficient for full GA₃-induced expression in aleurone protoplasts. In the intact aleurone layers, however, all three boxes seem to be required. Thus, the elements that constitute the GA

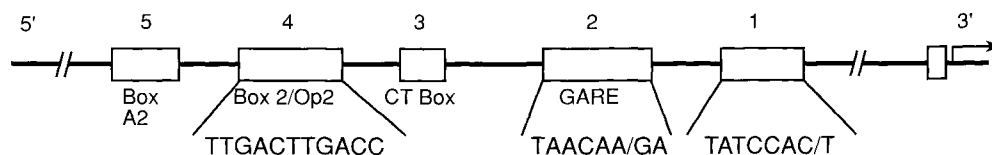


FIGURE 24-3 Functionally important *cis* elements in high *pI* and low *pI* α -amylase genes. High *pI* α -amylase genes have three elements: a TAACAA/GA element, which is the GA response element, a distal pyrimidine box with a preponderance of CT bases, and a proximal TATCCAC/T box. Low *pI* α -amylase genes have several other boxes 5' upstream of the pyrimidine box, including box 2 (which includes Opaque 2 element) and box A2. Sequences are numbered from 1 to 5 starting from the 3' end. Adapted from Huttly and Phillips (1995) and Wilmott *et al.* (1998) with kind permission from Kluwer.

response complex (GARC) vary depending on the tissue context, i.e., whether isolated protoplasts or intact aleurone layers are used. In low *pI* α -amylase genes, GARE, together with box 2 (sequence #4 in Fig. 24-3), is sufficient for high expression.

4.1.2. *cis* Elements in Other GA-Regulated Genes in the Aleurone System

Considerably less is known about the *cis* elements in other GA-regulated genes in the aleurone system. The promoter of a barley cysteine endoproteinase gene, *EPB-1*, has a GARE, a pyrimidine box, and an upstream element, which are required for high-level GA-induced expression. The barley (1-3, 1-4)- β -glucanase promoter also contains a recognizable GARE motif. However, the promoters of many other genes, such as the aleurain (a protease) from barley, a wheat carboxypeptidase, and, conspicuously, rice α -Amy3 genes, do not possess the TAACAA/GA sequence.

4.1.3. Transcription Factors

Specific binding of nuclear proteins to *cis* sequences referred to in Section 4.1.1 has been shown by *in vitro* footprinting, and a few transcription factors have been identified.

As mentioned in Appendix 1, MYB-type transcription factors in plants contain two conserved amino acid repeats (R2 and R3) that are important for DNA binding (see Fig. A1-10E). Since the TAACAA/GA element had some similarity to the DNA-binding sequence in MYB-type proteins, Jake Jacobsen and associates at CSIRO, Canberra, Australia, used an oligonucleotide probe containing parts of the R2 and R3 coding sequences to fish out a clone from a cDNA library from barley aleurone and then used polymerase chain reaction to obtain a full-length cDNA clone, which they called *GAmyb* (henceforth called *GA-MYB*). *GA-MYB* has been shown to be a transcriptional regulator of high *pI* α -amylase, as well as several other GA-induced genes, including the *EPB-1* gene, in barley aleurone. It binds to a DNA sequence containing the GA response element *in vitro*, specifically to the core-AAC- in the TAACAA/GA sequence. Its mRNA is induced by GA₃ treatment of aleurone layers prior to induction of α -amylase mRNA (Fig. 24-4) and is not inhibited by cycloheximide, which suggests that *GA-MYB* is a primary response gene.

Moreover, the constitutive expression of *GA-MYB* in transgenic plants can activate GA-induced genes, such as α -amylase or cysteine proteinase (*EPB-1*) genes, in the absence of exogenous GA (Fig. 24-5), a characteristic exhibited by many transcription factors.

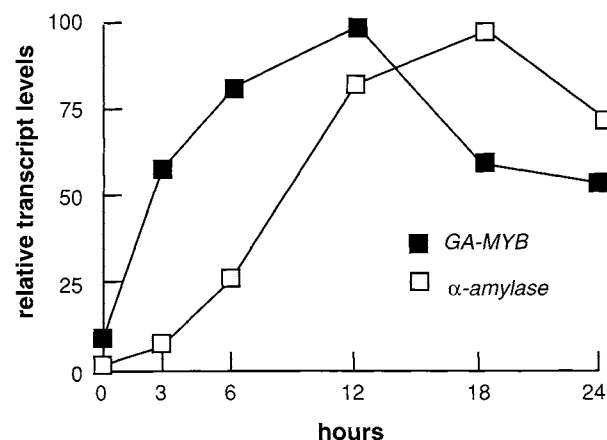


FIGURE 24-4 Time course of *GA-MYB* and α -amylase gene expression in GA₃-treated barley aleurone layers. RNAs were isolated at specified times and hybridized with gene-specific probes. The hybridization signal was normalized to that of rRNA before being plotted. From Gubler *et al.* (1995).

Plants have many MYB genes, including *C1*, which is involved in regulating anthocyanin biosynthesis in maize kernels (see Chapter 23). It could be shown in the experiment just described that only *GA-MYB*, and not *C1*, could activate the *AMY::GUS* construct.

Some other proteins that bind to the GARE or other *cis* sequences have been identified, but their precise roles are still unclear. Their relationship to *GA-MYB* is also unknown.

Data currently available on *cis* sequences in α -amylase genes and nuclear proteins that bind to them are summarized in Fig. 24-6.

4.2. Gene Regulation in Stem Elongation and Flowering

As mentioned in Chapter 15, genes for α - and β -tubulin, as well as for a water channel protein in tonoplast, γ -TIP, have been shown to be upregulated during GA-induced stem elongation, but how GA regulates their transcription is unknown.

Flowers of many plants accumulate anthocyanins in their petals (corolla), which are responsible for their brilliant colorations. The synthesis of anthocyanins is regulated by a variety of factors, including light, temperature, pathogens, and hormones. The development and pigmentation of petals in flowers of *Petunia hybrida* are apparently under the control of gibberellins. If stamens are surgically removed from *Petunia* flowers at a young stage, the corolla develops poorly and lacks pigmentation. However, if the young, emasculated flowers are treated with or placed in a solution of GA₃, corolla development and pigmentation are

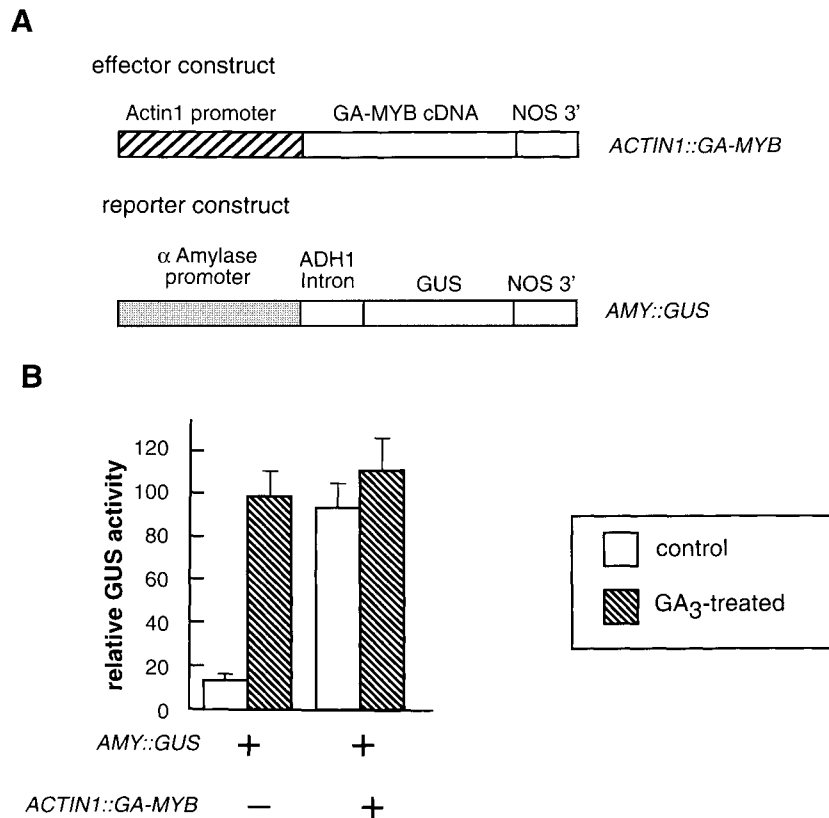


FIGURE 24-5 Transactivation of the high *pI* α -amylase promoter by GA-MYB in barley aleurone layers. (A) Two constructs were made: one construct consisted of an Actin1 promoter plus the GA-MYB-coding sequence (*ACT::GA-MYB*) and the other, a reporter construct, consisted of the proximal region of the α -amylase promoter fused to the GUS-coding sequence (via an intron from an alcohol dehydrogenase gene). Both constructs had a 3' end from the *nos* gene of *Agrobacterium* T-DNA. Barley half-grains were cocomm-barded with the two constructs, and GUS expression was monitored without (control) and plus GA₃ treatment. (B) A comparison of the control bars shows clearly that the *ACT::GA-MYB* construct by itself, in the absence of exogenous GA₃, is sufficient to raise the expression of the reporter gene by about 10-fold, an expression that is not increased any further by exogenous GA₃. Modified from Gubler *et al.* (1995).

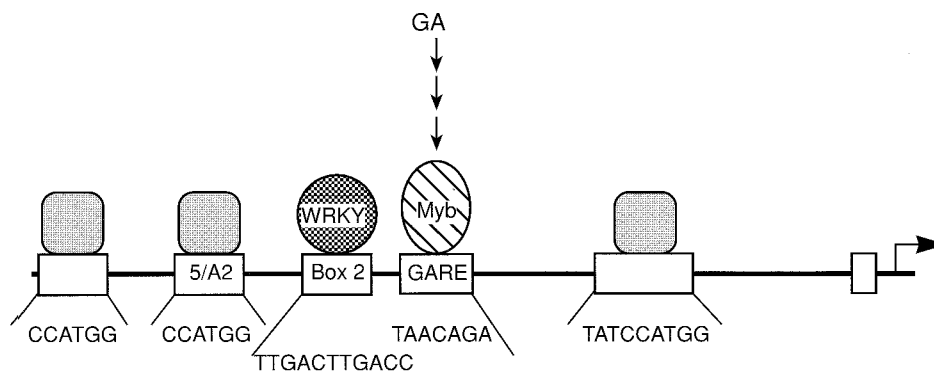


FIGURE 24-6 A summary of current data concerning transcription of high *pI* and low *pI* α -amylase genes in aleurone cells of cereal grains. Two transcription factors, a MYB type and a WRKY type, are shown bound to GARE and box 2, respectively. In low *pI* α -amylase genes, the GA response complex includes the GARE and box 2, whereas in high *pI* α -amylase genes, it includes the GARE and the more proximal *cis* element. Proteins whose identity is unknown are shown bound to the TATCCAC/T box and to sequences distal to box 2. Adapted from Wilmott *et al.* (1998) with kind permission from Kluwer.

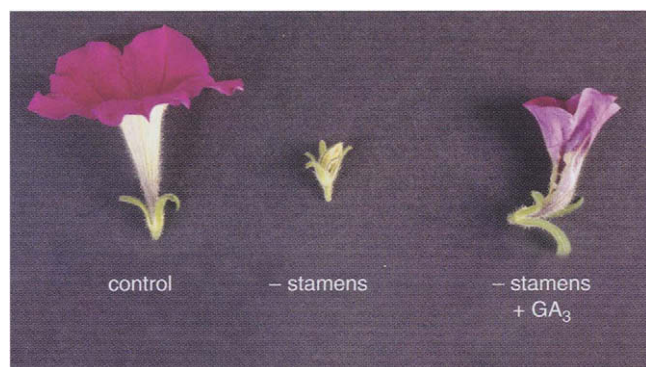


FIGURE 24-7 Effect of GA_3 on the development of corolla and accumulation of anthocyanins in *Petunia*. Courtesy of David Weiss, The Hebrew University of Jerusalem, Rehovot, Israel.

normal (Fig. 24-7). These observations suggest that corolla development and pigmentation are regulated by gibberellins, which are synthesized in the stamens. In support, GA synthesis genes have been shown to be expressed in young stamens. Some 15 structural genes encoding various enzymes mediating anthocyanin biosynthesis are coordinately expressed; some are expressed early, whereas others are expressed later, and GA has been shown to be involved in the expression of both sets. Although *cis* elements in promoters of many of these genes are known, there is little information on their GA-induced regulation. Apparently, Myb-type transcription factors are involved, and some are induced very early in response to GA and their synthesis does not require prior protein synthesis.

In Chapter 7, it was mentioned that *GA20 OXIDASE* genes in *Arabidopsis* and some other plants are regulated negatively by biologically active GAs. An analysis of their regulatory *cis* sequences and transcription factors that bind to them is likely to add substantially to our knowledge of GA-induced gene expression.

5. GA RESPONSE MUTANTS

GA response mutants fall into two phenotypic categories: GA-insensitive dwarfs and elongated slender mutants (see Table 24-2).

Dwarf mutants have a phenotype similar to that of weak or “leaky” alleles of GA-deficient biosynthesis mutants, except that exogenous treatment with GA does not restore them to the wild type. This class includes the well-known *Rht* (for reduced height) wheat lines used to develop the high-yield, short-statured wheat varieties that led to the “green revolution” in countries like Mexico and India (Fig. 24-8A). Other members include *dwarf8* (*d8*) maize and *GA-insensitive* (*gai*) *Arabidopsis*. Mutant alleles identified for these GA-insensitive dwarfs are all dominant or semidominant over the wild type and have pleiotropic effects. For instance, the *Rht3* mutant in wheat in the homo- or heterozygous state shows reduced stem height and leaf length compared to the wild type, and the aleurone does not produce α -amylase or α -amylase mRNA in response to GA_3 . The pleiotropic nature of these

TABLE 24-2 Selected GA Response Mutants^a

Plant	Mutant ^b	WT gene (nature of encoded protein)	Ref.
Dwarf phenotype			
<i>Triticum aestivum</i> (wheat)	reduced height (<i>Rht</i>) (sd)	<i>rht-1</i> (transcription factor)	Gale and Yousefian (1985), Peng <i>et al.</i> (1999)
<i>Zea mays</i> (maize)	dwarf8 (<i>d8</i>) (sd)	<i>D8</i> (transcription factor)	Peng <i>et al.</i> (1999), Harberd and Freeling (1989)
<i>Arabidopsis thaliana</i>	gibberellic acid-insensitive (<i>gai</i>) (sd)	<i>GAI</i> (transcription factor)	Peng <i>et al.</i> (1997)
<i>Silene armeria</i>	dwarf <i>Silene</i> (r)	—	Suttle and Zeevaart (1979)
<i>Oryza sativa</i> (rice)	<i>dwarf1</i>	α subunit of a heterotrimeric G protein	Mitsunaga <i>et al.</i> (1994)
Slender phenotype			
<i>Hordeum vulgare</i> (barley)	slender (<i>sln</i>) (r)	—	Foster (1977)
<i>Oryza sativa</i> (rice)	slender (<i>sln</i>) (r)	<i>SLN</i> (transcription factor)	Ikeda <i>et al.</i> (2001)
<i>Pisum sativum</i> (pea)	<i>la crys</i> double mutant (r)	—	Potts <i>et al.</i> (1985)
<i>Lycopersicon esculentum</i> (tomato)	procera (<i>pro</i>) (r)	—	Jones (1987)
<i>A. thaliana</i>	spindly (<i>spy</i>) (r)	<i>SPY</i> (a GlcNAc transferase)	Jacobsen <i>et al.</i> (1996)

^aIn wheat, the wild-type gene is written with lowercase letters, whereas for mutant alleles, the first letter is written in uppercase.

^bDominance: sd, semidominant or dominant alleles, r, recessive.



FIGURE 24-8 (A) Near-isogenic lines of wheat seedlings containing different reduced height (*Rht*) dwarfing genes in the background tall (*rht*) variety, Maris Huntsman (for usage of *Rht* and *rht*, see legend for Table 24-2). Modern bread wheat is hexaploid ($2n = 42$), containing A, B, and D genomes derived from three diploid ancestors, each containing seven pairs of homoeologous chromosomes. The semidwarfing genes, *Rht1* and *Rht2*, are present on chromosomes 4BS and 4DS, respectively. The more potent dwarfing gene, *Rht3*, is an alternative allele at the same locus as *Rht1*, and the combination of *Rht2* + *Rht3* leads to an even more severe dwarf phenotype. The extent of the block to responsiveness to applied GA is proportional to the degree of dwarfism with the semidwarf lines *Rht1* and *Rht2* showing slight GA responsiveness, whereas the more extreme dwarf lines, *Rht3* and *Rht2* + *Rht3*, are nonresponsive to the hormone. Courtesy of John Lenton, ICAR, Long Ashton Research Station, Bristol University, UK. (B) Wild-type and slender barley seedlings. *sln* Seedlings are homozygous segregants in the progeny of heterozygous plants. Courtesy of Peter Chandler, CSIRO, Canberra, Australia.

mutants suggests that they act at early steps in GA perception/signal transduction. The endogenous content of biologically active C_{19} GAs in these dwarf mutants is higher than in the wild-type plants, possibly because they are unable to regulate GA synthesis by feedback inhibition of GA 20-oxidases involved in GA biosynthesis (see Chapter 7).

In contrast to GA-insensitive dwarfs, slender mutants behave as if the GA response pathway is constitutively activated. All are recessive mutants, but there are differences between them. *sln1* Homozygous barley plants have elongated stems and leaves (see Fig. 24-8B), a constitutive synthesis and secretion of α -amylases and other enzymes in aleurone tissue, and are male sterile. The supply of exogenous GA or treatment with GA synthesis inhibitors has no effect on the *sln* phenotype. The *la cry*^s double mutant in pea, likewise, is unaffected by GA levels, but shows a slender phenotype only in the double mutant; individual alleles at the two loci, even in the homozygous state, show the wild phenotype. The *spy* (*Arabidopsis*) and *pro* (tomato) mutants partially respond to exogenous GA. In contrast to dominant or semidominant dwarfs, recessive mutants show a lower endogenous content of biologically active C_{19} GAs than the isogenic wild-type plants. The explanation seems to be that constitutive GA signaling represses GA 20-oxidase and, hence, synthesis of active GAs. Constitutive signaling is also likely to activate GA 2-oxidase, thus lowering the concentration of active GAs still further.

Many of the mutants just described are in species (wheat, barley, pea) that do not lend themselves easily to map-based cloning of genes and genetic manipulation. Hence, molecular genetic work, until very recently, had been done using *Arabidopsis*. This situation is changing, however, and homology-based cloning using information from the rice genome sequencing programs is being used to isolate genes from more intractable species such as wheat. In the following, we first consider some mutants isolated in screens for GA signaling in *Arabidopsis*, their wild-type genes and nature of their encoded proteins, and their orthologs in wheat and maize. We next consider some recessive mutations and their genes and encoded proteins before summarizing our present understanding of GA signaling.

5.1. *GAI* Locus

The *gai* mutant of *Arabidopsis* was isolated following X-ray irradiation of wild-type seeds. Genetic analysis indicated that it was a single gene semidominant mutation. The phenotype of *gai* is similar to that of GA-deficient *gai1* mutants, but there are some important differences. Both are dwarf, have darker green foliage, and take longer to flower than the wild type, but the *gai* alleles produce fertile flowers, whereas the severe mutant alleles at the *GAI1* locus show a drastic curtailment of inflorescence and flower development, especially petals and anthers (Fig. 24-9). The GA

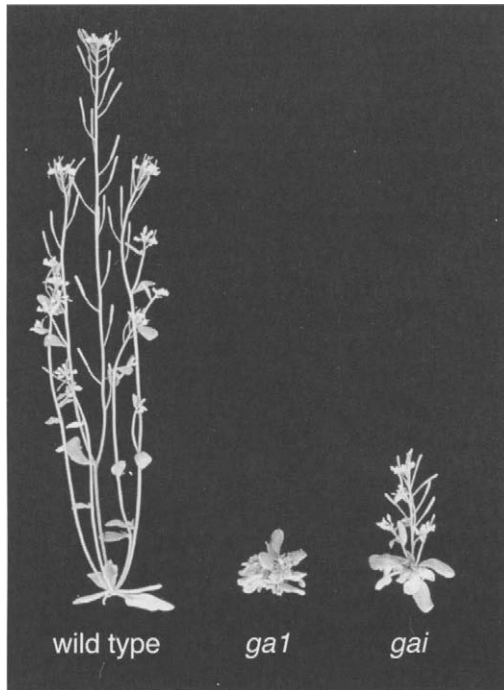


FIGURE 24-9 Wild-type *Arabidopsis* (Landsberg “erecta”), a severe GA-deficient synthesis mutant, *ga1-3*, and a GA-insensitive mutant, *gai*. Note that *ga1-3* is a dwarf and shows a curtailment of inflorescence and flowers; but *gai*, although a dwarf, shows inflorescence. From Koornneef *et al.* (1985).

insensitivity of *gai* alleles is quantitative, they are not completely unaffected by GA, and still retain a low level of GA responses.

Figure 24-9 illustrates an important point about GA-insensitive mutants. The GA insensitivity, depending on mutant alleles, may be well pronounced in vegetative parts, but not extend to reproductive parts. In some of the *Rht* dwarfs in wheat, such as *Rht1* and *Rht2*, which represent different genetic loci, the GA insensitivity is seen in vegetative parts, but not in reproductive axes or in aleurone tissue. In the more severe *Rht3*, which is allelic to *Rht1*, it extends to aleurone tissue as well. Likewise, in mutants of *Silene armeria*, GA insensitivity does not extend to flowers.

The *GAI* gene was cloned using a *Ds* transposon tag and encodes a protein that belongs to a novel class of plant transcription factors (see Sections 5.2 and 5.3). Comparison of *GAI* and *gai* sequences indicates that the *gai* gene has a 51-bp in frame deletion in the *GAI* open reading frame. As a result, the encoded gene product lacks a segment of 17 amino acids near the N-terminal, known as the DELLA region, named after the first 5 of the 17 amino acids (see Fig. 24-10). The *gai* protein is still produced, but its function is altered.

5.1.1. Loss-of-Function *GAI* Alleles Confer a Tall Phenotype

Since the *gai* mutation is dominant, it could not be concluded definitively how the wild type (*GAI*) or the mutated protein interacts with the GA response pathway. Hence, loss-of-function mutations at the *GAI* locus were created by γ -irradiation and screening for suppression of the *gai* phenotype (*gai-d* mutant); other loss-of-function mutants were available from the *Ds* transposon tagging used to clone the *GAI* gene (e.g., *gai-t6*). These loss-of-function mutations yield tall plants, which, in their phenotype, resemble the wild-type plants (Fig. 24-10). Sequence analysis indicates that the mutations are intragenic and interrupt the open reading frame of the *gai* gene; hence, they are unlikely to encode functional products.

Since a loss of function at the *GAI* locus confers a phenotype that is similar to the wild type, it may be concluded that *GAI* is dispensable for the control of growth in normal conditions. However, this is not strictly true. These loss-of-function mutants also show an insensitivity to paclobutrazol, a GA biosynthesis inhibitor (see Chapter 7). At the same concentration of paclobutrazol, mutant plants show greater stem growth and more open flowers and extended petals than the wild type (Fig. 24-11). This increased resistance to paclobutrazol indicates that *GAI* is involved in the wild-type GA response, perhaps as a negative regulator of GA-mediated growth responses.

5.2. *GRS* and *RGA*: Genes Similar to *GAI*

Arabidopsis contains another gene, *GRS* (for *GAI*-related sequence), which encodes a product (*GRS*) very similar to *GAI*. Moreover, the *GRS* gene has been found to be the same as *RGA* (see later).

As explained in Chapter 7, the *GAI* locus encodes the first enzyme in GA biosynthesis, copalyl synthase, which catalyzes the conversion of geranylgeranyl diphosphate to copalyl diphosphate. To know more about GA signaling, extragenic suppressor mutants for the GA-deficient, *ga1-3* phenotype (see Fig. 24-9) were generated. Several mutant alleles were obtained, including some at a previously identified *SPY* locus (see Section 5.6). Other mutant alleles, designated *rga* (for repressor of *ga1-3*), defined a new locus, *RGA*. The *rga/ga1-3* mutations are recessive; they enhance the stem growth of *ga1-3* to 30–50% of wild-type levels, but still retain partial male sterility (Fig. 24-12). GA-deficient mutants contain elevated levels of *GA4* transcripts (encoding 3 β -hydroxylase, which converts C₁₉ GAs to active forms). The *rga* mutants are deficient in endogenous GA, but, unlike GA-deficient mutants,

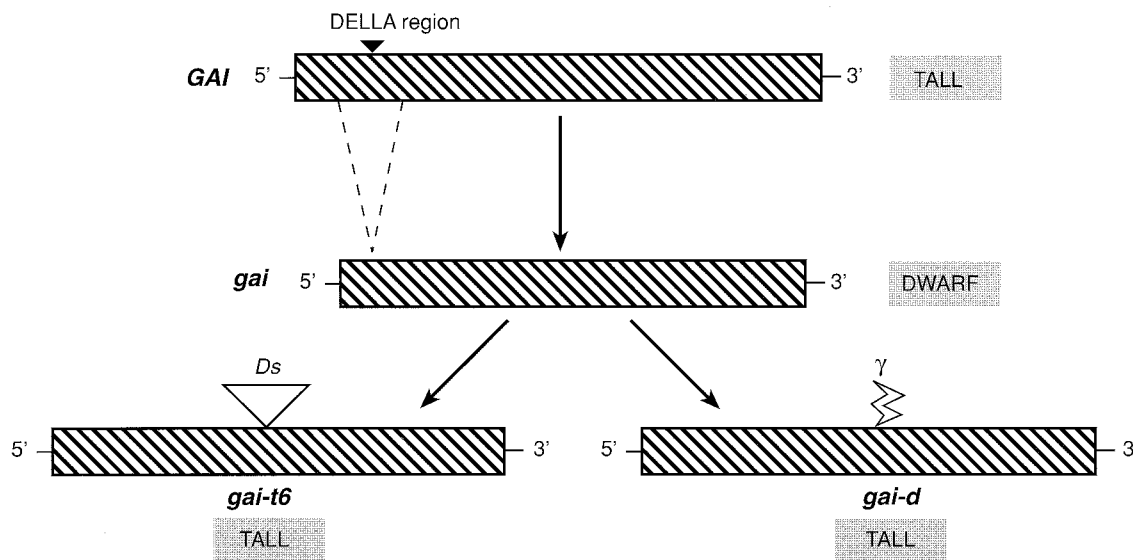


FIGURE 24-10 Schematic representation of *GAI*, dominant *gai* and loss-of-function alleles, *gai-t6* and *gai-d*, and their phenotypes. From Harberd *et al.* (1998) and Peng *et al.* (1997).

suppress the levels of *GA4* transcripts. Hence, it is thought that the RGA protein acts in the GA response pathway. Moreover, their deficiency in endogenous GA content, coupled to a partial increase in stem growth, suggests that plants lacking RGA require less GA than normal plants for equivalent growth. Therefore, RGA, like GAI, opposes the effect of GA and functions as a negative regulator of GA-mediated growth responses.

Although GAI and RGA both seem to be redundant to normal growth, they are expressed throughout the plant.

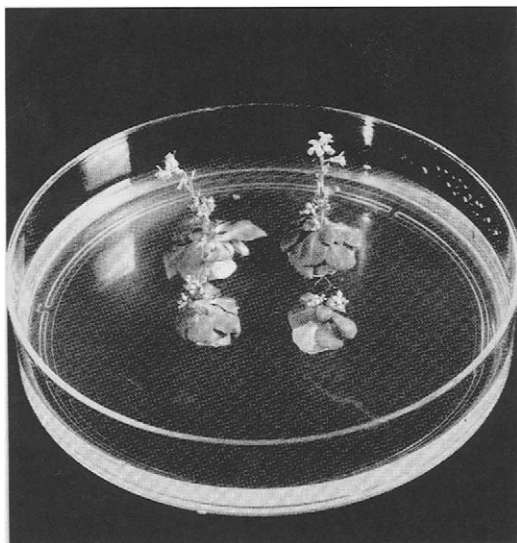


FIGURE 24-11 Growth inhibition in *GAI* (two plants in front) and *gai-t6* (two plants in rear) grown on media supplemented with paclobutrazol (10^{-6} M). Inflorescence stem growth is suppressed in the wild type, but not to the same extent in the mutant. From Peng *et al.* (1997).

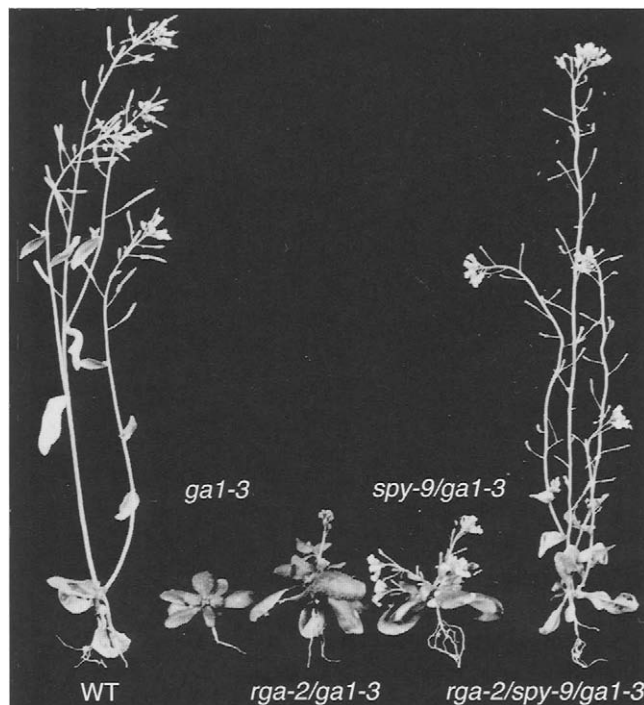


FIGURE 24-12 Phenotype of *Arabidopsis* wild type (Landsberg erecta, Ler), *rga*, and *spindly* (*spy*) mutants in *gal-3* background. Note that *rga* and *spy* both enhance shoot growth over *ga1-3* and that the *rga/spy* combination restores *gal-3* to almost the wild phenotype. From Silverstone *et al.* (1997).

The *RGA* gene was cloned and found to be the same gene as *GRS*. *GAI* and *RGA/GRS* (henceforth called only *RGA*) belong to a novel gene family, which was first defined by *SCARECROW* (*SCR*) in *Arabidopsis*. *SCR* encodes a putative transcription factor, which regulates the pattern of cell divisions regulating endodermis development in *Arabidopsis* roots and stems. Members of this gene family, designated GRAS family (acronym for *GAI*, *RGA*, *SCR*), occur extensively in plants. Nineteen members are known from *Arabidopsis* alone (in addition to the 3 just described, 16 others are known from expressed sequence tags). Orthologs of *SCR* are known from maize and pea. Another member, *LATERAL SUPPRESSOR* (*LS*), is known from tomato where it encodes a protein thought to regulate the origin of axillary branches at the vegetative shoot meristem (see Chapter 14). So far, members of this family have been found in plants only.

5.3. Structure of GAI/RGA Proteins

The GRAS family of proteins shows a variable N-terminal region, but a common C-terminal region with several conserved domains. These domains include two leucine heptad repeat regions (LHR1 and LHR2), a highly conserved VHIID domain, which typifies the family, and two domains designated PYFRE and SAW toward the carboxy terminus (Fig. 24-13). The sequence of these domains, although not the spacing between them, is highly conserved. The functions of most of these domains are still unknown, although the LHR regions are thought to be involved in protein-protein interactions and formation of multimeric complexes.

GAI/RGA differ from most of the GRAS family members in their N termini. Both have two regions of closely related sequence (regions I and II, the first five amino acids of region I are DELLA; hence, this region is referred to as the DELLA sequence). These regions are lacking in other members of the GRAS family,

including *SCR* and *Ls*, which indicates that these regions are probably important for GA signaling, a conclusion supported by the fact that mutations in this region render plants insensitive to applied or endogenous GA (e.g., *gai* mutant). In common with *SCR*, they contain nuclear localization signals (NLSs). Moreover, a fusion protein between *RGA* and green fluorescent protein (reporter) is localized to the nucleus in onion epidermal cells, indicating that the NLSs are functional.

5.4. *Rht1* and *Rhtd* Genes in Wheat and *d8* Maize Encode Functional Orthologs of GAI/RGA

The dominant or semidominant mutants, *Rht* (for reduced height) wheat and *dwarf8* (*d8*) maize, have been known for decades, but the cloning of their wild-type genes was difficult because, as indicated in the legend for Fig. 24-8, cultivated wheat is hexaploid with three homoeologous chromosome sets (the A, B, and D genomes), and isolating a maize gene *via* gene tagging is a laborious process. Nicholas Harberd at John Innes Centre, Norwich, United Kingdom, and associates used a data base search of rice expressed sequence tags to identify a DNA sequence that could encode a polypeptide with the DELLA sequence of amino acids. Since cereal genomes are well characterized and show substantial conservation of gene order (colinearity), they could use the rice sequence to isolate a wheat cDNA and ultimately the wheat *rht* genes located on 4B and 4D chromosomes, as well as the maize *d8* gene. The encoded products of wheat *Rht1* and *Rht2* and maize *d8* genes show 88% amino acid identity and map to the same region of cereal genome; hence, they are thought to be the same gene.

The deduced amino acid sequences of the wild type wheat *Rht1* and maize *D8* proteins contain all the conserved domains in *GAI/RGA* proteins, including the N-terminal regions I (DELLA region) and II, the



FIGURE 24-13 Schematic structure of *GAI/RGA/SCR*-type proteins. Conserved domains in the carboxy terminus are designed by single letter code for amino acids. LHR regions are probably involved in protein-protein interactions, but the functions of other domains, VHIID, PYFRE, and SAW, are unknown. The N termini of *GAI* and *RGA* proteins differ from *SCR* in that they contain two closely related sequences—regions I (DELLA region) and II—which are thought to be specific for GA signaling. Adapted with permission from Pysh *et al.* (1999), © Blackwell Science Ltd.

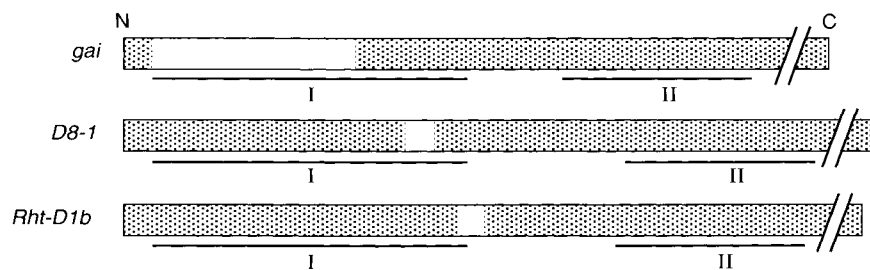


FIGURE 24-14 Schematic representation of the N-terminal regions of proteins encoded by dominant GA-insensitive mutant alleles: *gai* *Arabidopsis*, *D8-1* maize, and *Rht-D1b* wheat. The conserved regions, DELLA I and II, are underlined, and deletions (including substitutions) are highlighted in white. All mutations alter the N-terminal region of their encoded proteins. Modified with permission from Peng *et al.* (1999).

NLSs, and the other domains in the carboxy terminus. The semidominant mutations in wheat and maize, allelic to *Rht1* and *d8*, confer differing severities of dwarfism. Like *gai*, they are not completely insensitive to GA. For instance, maize *d8-1* mutants treated with paclobutrazol show enhanced dwarfism, which is corrected to original dwarf stature by simultaneous GA₃ treatment. Sequence analysis of these alleles indicates that they all contain mutations in the N terminus, particularly the DELLA region (Fig. 24-14), but seem to produce truncated proteins that are still active. Moreover a loss-of-function allele of wheat *Rht1*, which lacks the DNA sequence encoding the DELLA region, confers a tall phenotype, which is GA responsive. Thus, it is concluded that *Rht1* and *Rht2* wheat and *D8* maize are functional orthologs of *Arabidopsis* GAI/RGA.

5.5. *Slender (sln)* Mutants of Barley and Rice

Although the *sln* mutant of barley (see Table 24-2 and Fig. 24-8B) has been known for a long time, its gene has not yet been cloned. Recently, a *slender (sln1)* mutation in rice was obtained, it is a recessive mutation which, like its barley counterpart, shows a constitutive GA response; the plants are inordinately tall as if saturated with GA, insensitive to applied paclobutrazol, and their aleurone tissue produces α -amylase without an exogenous GA treatment. Since *sln1* is a loss-of-function mutation, the *SLN1* locus negatively regulates the GA responses. The *SLN1* gene was cloned and found to encode an ortholog of GAI/RGA/Rht/D8, with all the conserved domains including the DELLA sequence, an NLS, the leucine heptad repeats, and the VHIID domain. Several *sln1* alleles occur in rice. The *sln1-1* allele has a premature stop codon and is a null allele that does not produce a protein.

These data from *Arabidopsis*, wheat, maize, and rice indicate that GAI/RGA/Rht/D8/SLN represent a protein belonging to the GRAS family of transcriptional regulators, which is specific to GA responses. If the protein is mutated in the DELLA region it produces a defective protein which makes the system mostly, though not completely, GA insensitive. By contrast, if the protein is not expressed at all because of a null mutation, it gives a constitutive GA response. The data from *slender* rice also confirm the earlier results that loss-of-function intragenic suppressor mutations in the *GAI/Rht* genes result in a tall phenotype.

5.6. *SPY* Locus

Since seed germination in *Arabidopsis* depends on endogenous GA (more correctly, a balance between endogenous GA and ABA), GA-insensitive mutants can be isolated by screening for seeds that germinate under conditions of reduced endogenous GA. The *spy* (for spindly) mutations were obtained by mutagenizing seeds and plating them on a medium with 1 μ M paclobutrazol. Several independent recessive mutants were isolated; all were alleles at the *SPY* locus. As expected, these mutants have a reduced requirement for GA in seed germination. They also have longer internodes and display early flowering, partial male sterility, and increased parthenocarpy in fruit (siliques), traits shown by wild-type plants when supplied with an excess of biologically active GAs. However, *spy* mutants, unlike *slender* mutant in barley, still respond to exogenous GA, which suggests that they are not constitutive to GA responses. Since *spy* alleles are recessive, the *SPY* locus is postulated to encode a negative regulator of the GA response.

Double mutational analysis indicates that *spy* alleles partially suppress the phenotype associated with GA

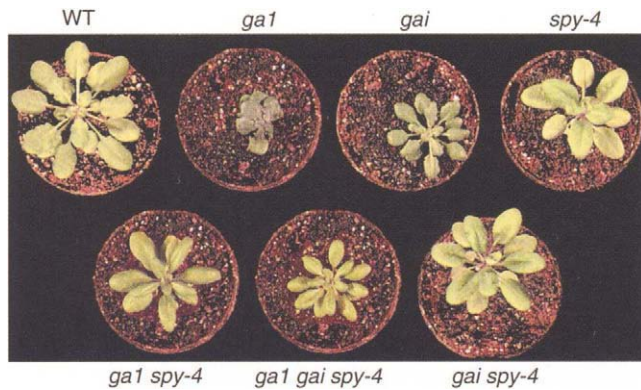


FIGURE 24-15 Phenotypes of adult wild type, *ga1*, *gai*, and *spy-4* mutants of *Arabidopsis*. The phenotypes of *spy-4* in *ga1*, *gai*, or *ga1 gai* backgrounds are also shown. *spy-4* is a strong allele and shows a near-normal phenotype; it also restores *ga1* and *gai* partially to wild type. Courtesy of Neil Olszewski, University of Minnesota, St. Paul.

deficiency (e.g., *ga1* alleles) or GA insensitivity (*gai* alleles) (Fig. 24-15). They also enhance the suppression of the *ga1-3* phenotype conferred by *rga* alleles (see Fig. 24-12). Mutant alleles at the *SPY* locus are epistatic to *rga* and *gai*, which suggests that *SPY* acts downstream of *RGA* and *GAI* or that it modifies those proteins.

The aleurone system is a well-characterized system for GA-induced gene expression. Accordingly, a barley homologue of *SPY*, *HvSPY*, was cloned. The coding sequence of *HvSPY* under a constitutive promoter was coexpressed in aleurone tissue along with a high *pI* α -amylase::GUS construct. The constitutive expression of *HvSPY* reduced the GUS expression in GA₃-treated aleurone layers, which confirms that *SPY* acts as a negative regulator of the GA response pathway (Fig. 24-16).

SPY and *HvSPY* genes are orthologs. They encode a protein that is structurally similar to a class of enzymes in animals that transfer *N*-acetylglucosamine to target proteins. These enzymes, known as O-GlcNAc transferases (OGTs), transfer a single GlcNAc from UDP-GlcNAc to specific serine and/or threonine residues via an O linkage. Such O-GlcNAc modification of regulatory proteins, similar to protein phosphorylation, is believed to play a role in signal transduction pathways. The *SPY* protein contains 10 copies of a tetrapeptide repeat (TPR) motif at the N-terminal and a C-terminal catalytic domain, both features seen in cytosolic OGTs from animal systems (Fig. 24-17). The TPR motif is involved in protein-protein interactions. Whether the *SPY* protein acts as an OGT in plant systems is unknown, but recent data suggest that it is involved in several roles in plant development including a negative regulation of GA responses.

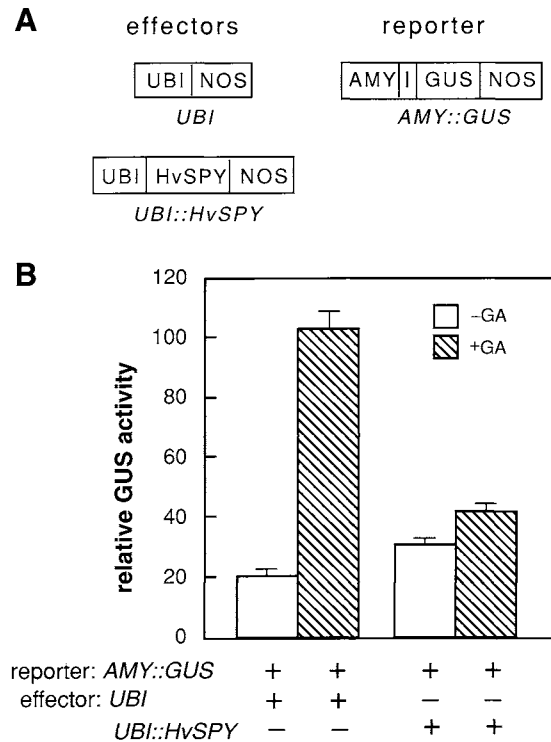


FIGURE 24-16 Functional analysis of *HvSPY* protein. (A) Structure of the constructs. Effector constructs were either a ubiquitin promoter fused to the *HvSPY*-coding sequence (*UBI::HvSPY*) or a blank cassette consisting of the *UBI* promoter only (control). The reporter construct consisted of a barley high *pI* α -amylase promoter fused, via an intron (I), to the GUS-coding sequence (*AMY::GUS*). Each construct was terminated with the 3' region of the *NOS* gene from *Agrobacterium*. (B) Barley half-grains were cobombarded with the reporter gene plus either of the two effector constructs and incubated in a medium plus or minus 10^{-6} M GA₃. Relative GUS activity is expressed as a percentage of total GUS activity in aleurone layers bombarded with the blank *UBI* cassette and the reporter gene construct. The means and standard error are shown. From Robertson *et al.* (1998).

5.7. Other Mutant Loci

Other mutant loci identified in *Arabidopsis* include *SLEEPY* (*SLY*) and *PICKLE* (*PKL*). Recessive *sly1* mutations were identified in a suppressor screen for an ABA-insensitive, *abi1-1*, mutation. The mutants have a reduced GA requirement for seed germination. They also have a phenotype similar to that of GA-deficient mutants, but it is not corrected by exogenous GA. The recessive *pk1* mutations, identified in a screen for affected root development, show some features reminiscent of GA-deficient or -insensitive mutants combined with primary roots, which have swollen tips and retain the characteristics of an embryonic root. These loci await further characterization before their role in GA signaling can be assessed.

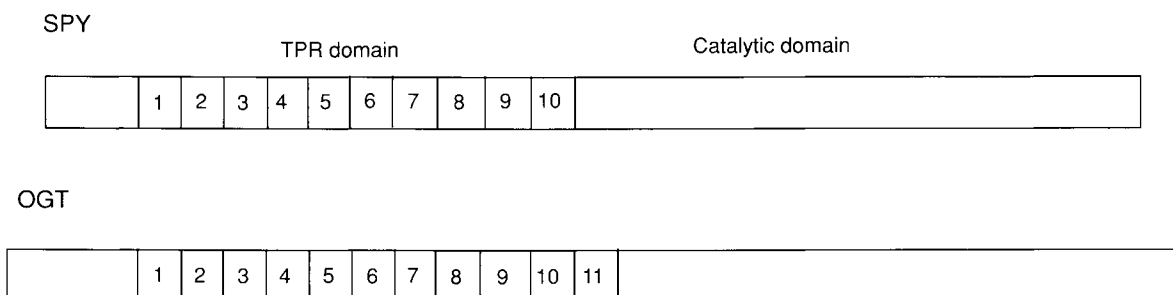


FIGURE 24-17 Schematic representation of *Arabidopsis* SPINDLY (SPY) protein. From Thornton *et al.* (1999) with permission from Elsevier Science.

6. A WORKING HYPOTHESIS FOR THE GA RESPONSE PATHWAY

Data reviewed so far suggest a working hypothesis for GA signaling with several unconnected pieces in the puzzle. The balance of evidence favors a cell surface perception of the GA signal by a transmembrane receptor, which could be a receptor-like kinase (RLK) or a G-protein-coupled receptor. The downstream elements for signaling are unknown. For RLK-type signaling, a type 2C protein phosphatase may be involved; for the G-protein-coupled receptor, secondary messengers (e.g., cyclic GMP, Ca^{2+}) and Ca^{2+} -calmodulin-activated protein kinases have been postulated.

Molecular genetic data from response mutants in *Arabidopsis*, wheat, maize, and rice indicate that GA responses are negatively regulated by a protein or a protein complex. A model for GA signaling is shown in Fig. 24-18. According to the model, elongation growth *via* the GA response pathway is repressed by an inhibitor protein represented in Fig. 24-18 by GAI (it could also be RGA/Rht/D8/SLN). This repression is relieved when endogenous or supplied GA is present at a concentration above a threshold. A mutation in the N-terminal DELLA sequences in the repressor protein confers a dominant phenotype; an altered function protein is produced which cannot be derepressed by GA, thus providing GA-insensitivity. By contrast, a loss-of-function mutation in the gene, which results in a lack of the protein, allows constitutive GA responses. Additional studies indicate that not only elongation growth, but most GA-regulated processes are controlled through a negatively acting GA-signaling pathway. The concept that plants contain an endogenous inhibitor of growth and that GA acts by inhibiting the activity of this inhibitor is not new. In fact, it was proposed by Percy Brian in 1957.

The position of SPY in this model is unclear. It could modulate GAI/RGA activity by the addition of *O*-linked GlcNAC residues or modulate the activity of some other protein downstream of GAI and RGA. It may also be part of an alternate signaling pathway that converges with the GA-signaling pathway.

In Fig. 24-18, a single protein is shown as the inhibitor, but it could also be a complex of several proteins including GAI/RGA or their orthologs in other plants. An altered function protein produced by *gai/rga* "poisons" the complex rendering it insensitive to GA, whereas a lack of the protein allows constitutive GA signaling. A negative regulation of the GA response pathway is somewhat similar to ethylene signaling (see Chapter 21). In ethylene signaling, ethylene binding to its receptor(s) derepresses the ethylene signaling pathway. However, GAI/RGA and, by analogy, their orthologs in wheat, maize and rice are not plasma membrane-based receptor proteins, like ETR1. Structurally, they contain no motifs, which suggest an association with membranes; instead, they are localized to the nucleus and have the hallmarks of transcription activators. Their biochemical functions and target genes are unknown. They could regulate transcription factors such as GA-MYB, which, in turn, could, lead to GA-specific gene expression, but such regulation still has to be shown. Their upstream signaling elements are also unknown.

The cereal aleurone system has long been considered a paradigm for hormone-mediated gene expression. GA signaling in this system has been associated with surface perception of the GA signal, participation by heterotrimeric G proteins, and secondary messenger-activated protein kinases. The availability of Rht in wheat and SLN in rice, makes it possible to test the roles of these proteins in GA signaling in the aleurone system. In this connection, it has been shown already that overexpression of *GAI* or *gai* in rice suppresses the GA-induced α -amylase production, while lack of SLN expression in rice allows

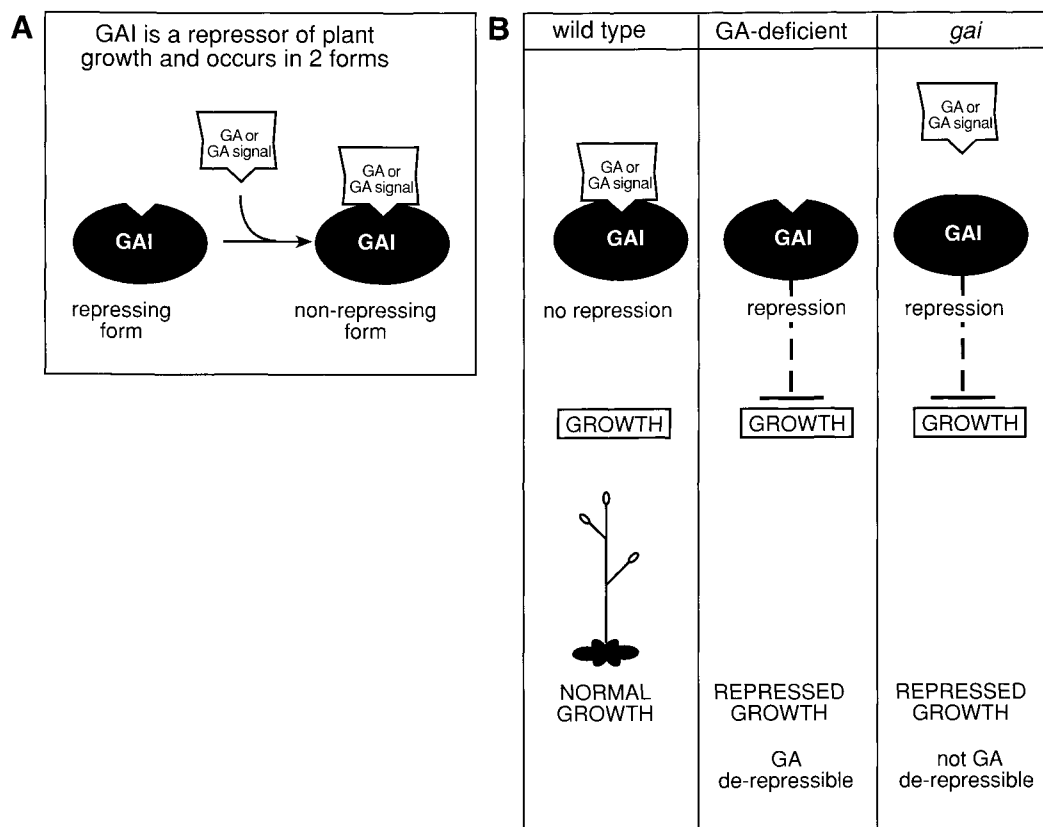


FIGURE 24-18 Derepression model for regulation of plant stem elongation by gibberellin (GA). GA (possibly *via* a signaling intermediate) opposes the activity of GAI, a protein that represses plant growth. (A) GAI exists in two forms. Interaction between GAI and GA (or the GA signal) results in conversion of the growth-repressing form of GAI into a nonrepressing form. (B) Normal (wild-type) plants grow tall because endogenous GA converts GAI to the nonrepressing form. GA-deficient plants are dwarfed because they lack sufficient GA to allow conversion of GAI, which therefore remains in the repressing form. *gai* Mutant plants are dwarfed because the mutant *gai* protein cannot interact with GA (or GA signal) and thus is not converted to the nonrepressing form. Reprinted with permission from Harberd *et al.* (1998).

constitutive α -amylase production. The wheat system is particularly instructive because *rht* alleles offer different degrees of GA insensitivity. *Rht1* and *Rht2*, which represent different genetic loci, are GA insensitive in vegetative parts, but not in aleurone tissue, whereas *Rht3* (allelic to *Rht1*) shows GA insensitivity in the aleurone tissue as well.

7. GA/ABA INTERACTION

Many, although not all, GA-induced responses are antagonized by ABA, and *vice versa*. For instance, GA promotes cell division and cell elongation in young growing tissues. Associated with cell division, it stimulates the accumulation of *cdc2* mRNA and some cyclins, both of which are inhibited by ABA. Cell elongation is accompanied by a predominantly

transverse orientation of microtubules in the parietal cytoplasm and enhanced activities of xyloglucan endotransglycosylases (XETs) that play a role in wall loosening. GA promotes these processes, while ABA inhibits them. Leaf development in aquatic plants is affected in opposite ways by GA and ABA. Leaves initiated while the shoot apex is underwater are dissected and filiform, whereas those initiated when the shoot apex is in air tend to be entire or much less lobed (see Fig. 1-2 in Chapter 1). GA supplied to leaves, which are developing while submerged, accentuates the dissected and filiform morphology, whereas ABA given under similar circumstances promotes the development of an aerial type leaf. ABA induces seed dormancy and can inhibit germination, whereas GA promotes seed germination and may be involved in breaking seed dormancy. A graphic demonstration of ABA/GA antagonism is seen in the induction of genes in the cereal aleurone system. GA induces the tran-

scription of α -amylase and several other protease and nuclease genes and suppresses the expression of the α -amylase inhibitor, alcohol dehydrogenase, and some other genes. ABA has the opposite effect.

The question arises — where does this antagonism occur? Do the two hormones interfere with each other at the receptor level, in the signaling pathway, or at the gene transcription level? It does not seem likely that the two hormones interfere with each other at the receptor level. *In vitro*-binding assays using [3 H]GA₁ or [3 H]GA₄ show no competition by ABA. Also, examples there are that the two signaling pathways can proceed independently of each other. For example, ABA induction of anion current in the guard cells is not interfered with by GAs. In the *slender* mutant of barley, which is a constitutive GA responder, α -amylase gene expression is inhibited by ABA, but ABA- and dehydration-induced gene expression in roots remains unaffected.

Gene transcription in the aleurone system is affected by both GA and ABA. Hence, the promoters of genes induced by GA or by ABA in this system have been studied for clues. As mentioned earlier, the ABA and GA response elements are different and presumably bind different transcription factors. The response elements work in concert with other *cis* elements with which they form response complexes. At least one *cis* element, Opaque2, in a low pI α -amylase promoter in barley, is able to form a response complex with either an ABRE or a GARE and be responsive to either ABA or GA, respectively. Thus a reasonable postulate would be that ABA acts on the regulated gene by interfering with the binding of transcription factors to GARC. It is unlikely that ABA *per se* causes this interference, rather it would be some component of the ABA signal transduction that would so interfere.

There are indications that such interference by a component in ABA signaling occurs further upstream on the GA signaling pathway. For instance, GAMyb constitutively expressed is sufficient to drive the expression of α -amylase and several other genes, including the gene for a cysteine proteinase in barley (*EPB-1*). While ABA suppresses the GA-induced gene expression, it does not suppress the GAMyb-induced expression; hence ABA must operate at a level upstream of GAMyb, as shown schematically in Fig. 24-19A.

The role of protein kinases and protein phosphatases in signal transduction is discussed in Chapter 25. However, it is appropriate to mention here that a protein kinase that is specifically induced by ABA (PKABA1 for protein kinase responsive to ABA), if expressed constitutively in barley aleurone tissue, suppresses the GA-induced expression of low pI and high pI α -amylase genes, without exogenous ABA,

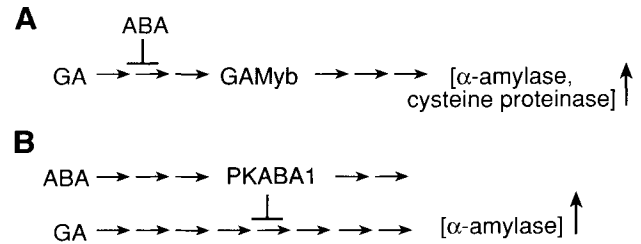


FIGURE 24-19 Schematic illustration of cross talk between GA and ABA signaling in cereal aleurone. (A) GA induces the synthesis of GAMyb, which, in turn, regulates the expression of GA-induced genes, such as α -amylase. ABA has no effect on GA responsive gene expression, however, if GAMyb is expressed constitutively. Hence, ABA inhibits GA signal transduction at a step before the synthesis of GAMyb. (B) ABA induces synthesis of a protein kinase, PKABA1, in wheat embryo. Constitutive expression of PKABA1 in barley aleurone inhibits GA-induced α -amylase gene expression, indicating that ABA mediates its effect on GA-induced gene expression *via* a protein kinase. Based on data in Cercós *et al.* (1999) and Gómez-Cadenas *et al.* (1999).

which suggests that ABA exercises inhibition over GA-induced responses via a protein kinase (Fig. 24-19B). Such constitutive expression of PKABA1 has little effect on expression of an ABA-specific LEA gene, *HVA1*, however, implying that *HVA1* is regulated by ABA in some other manner. Another ABA and salt stress-induced protein kinase in salt-tolerant wild wheatgrass, *Lophopyrum elongatum*, has been found to inhibit a GA-induced low pI α -amylase gene expression in barley aleurone tissue.

8. GA INTERACTION WITH ENVIRONMENTAL FACTORS

In addition to ABA, GAs are also known to interact with environmental factors, such as light and temperature. Seeds of many plants, while in a wet state, require an exposure to light in order to germinate. These seeds are referred to as photoblastic seeds. Some of the common examples include *Arabidopsis* and certain cultivars of lettuce (e.g., *Lactuca sativa* cv Grand Rapids). The phenomenon has been well studied for lettuce seeds, which have an absolute requirement for exposure to red light at 680 nm while imbibed. It is a phytochrome-regulated phenomenon and will be dealt with in Chapter 26. Here, it is important to point out that this red light requirement can be completely bypassed by exposure of seeds to a solution of GA. Similarly, many winter annuals, biennials, and perennials growing in temperate climates have a requirement for exposure to cold temperature, a phenomenon known as vernalization, before they will flower. Weeks and

months may elapse between the perception of temperature signal and the flowering response. Examples include winter wheat, winter rye, and biennials such as spinach or rape. In these plants, an exposure to exogenous GA has been shown to bypass the cold temperature requirement. While the molecular mechanisms by which GA bypasses these requirements are not understood, it is clear that the GA signaling pathway must intersect and cross talk with other signaling pathways related to phytochrome or cold temperature.

9. GA RECEPTOR(S) COULD BE A MEMBER OF A SMALL GENE FAMILY

It is possible that there are different isoforms of a GA receptor in different groups of plants, and also possibly for different responses, such as cell elongation, flowering, production of hydrolytic enzymes in seed tissue, and production of antheridia in fern gametophytes. Biological activities of C-3, 13-dihydroxy GAs, such as GA₁ or GA₃, and those of the C-3 single hydroxylated GAs, such as GA₄ or GA₇, differ in different families of plants. For example, GA₁ or GA₃ has higher activities in members of the legume and grass families than in cucurbits or *Arabidopsis*, whereas the reverse is true for GA₄ or GA₇. In ferns and many gymnosperms, GA₄ or GA₇ often has higher activities than GA₁/GA₃. In some long-day plants, such as *Lolium*, some polyhydroxylated GAs are more active in promoting flowering than in stem elongation. In contrast, GAs that are active in stem elongation, such as the monohydroxy GA₄, GA₇, or dihydroxy GA₁, GA₃ have very little promotive activity in flowering. The promotion of antheridia formation in gametophytes of certain ferns also involves a GA-type structure different from that of GAs that promote stem elongation. These observations are based on bioassays and are not conclusive, but they suggest that different isoforms of the GA receptor may be present among different taxa and possibly at different developmental stages. Such isoforms would represent different members of a small gene family.

10. SECTION SUMMARY

Hormone-binding studies have indicated the presence of soluble and membrane-bound proteins in GA responsive systems, but the proteins have not been purified and their identity remains unknown. There

is also substantial evidence for a cell surface perception of the GA signal by a G-protein-coupled receptor or a transmembrane receptor-like kinase. Studies on gene expression in the aleurone system have revealed many *cis* sequences in GA-regulated promoters, which seem to bind nuclear proteins. A MYB-type transcription factor specific to GA signaling mediates the expression of many GA responsive genes in the aleurone system and possibly other GA responses. Some other putative transcription factors are known but have not been characterized. The upstream signaling components that these transcription factors interact with are unknown. Molecular genetic studies using response mutants from *Arabidopsis*, wheat, and rice maize indicate that GA responses are negatively regulated by a protein or protein complex (e.g., GAI/RGA in *Arabidopsis* or their orthologs in wheat, and rice maize). The inhibitory protein(s) represses the normal response(s), and GA acts not by directly stimulating growth or another response, but by derepressing the inhibitor protein. GAI/RGA-type proteins are unique to GA signaling, but share structural motifs in their carboxy terminus, with other members of the GRAS family, a family of plant transcription factors of diverse functions. Other repressors of GA action, such as SPY in *Arabidopsis* and its ortholog in barley, are O-GlcNAc transferases, which may act by the modification of GAI/RGA-type proteins or some other proteins in the signal transduction pathway. GA interaction with ABA and environmental factors, such as light and temperature, underscores the complexity of signal transduction pathways, their interconnections, and possibilities of cross talk between them. Some data indicate that cross talk among GA and ABA-signaling pathways occurs in later steps of signal transduction, but prior to the synthesis of GA-specific transcription factors. There is increasing evidence for a role for G-protein signaling and of calcium and phosphorylations/dephosphorylations in early events in GA signaling. These aspects are covered in Chapter 25.

SECTION II. MOLECULAR BIOLOGY OF CYTOKININ (CK) ACTION

1. CK-INDUCED RESPONSES

The major effects of cytokinins on plant growth include the promotion of cell division in cell and tissue culture, promotion of lateral branching, inhibition of root growth, and delaying of senescence and abscission of leaves, petioles, and flowers. In dark-grown

seedlings, cytokinins promote expression of the photomorphogenetic program, i.e., inhibition of hypocotyl growth, differentiation of chloroplasts from proplastids, greening, and expression of *CAB* and *RBCS* genes. Cytokinins are also active in mosses (e.g., *Physcomitrella patens*), where they promote bud (gametophore) formation on protonema. Many of the effects of cytokinins, such as regulation of lateral bud growth and apical dominance, and root-shoot ratio in tissue culture are mediated in association with auxin. Higher cytokinin to auxin ratios promote shoot over root growth and promote outgrowth of lateral buds and a bushier habit.

2. CK-BINDING PROTEINS

Cytokinin-binding studies have been carried out using ^{14}C or ^3H -labeled N^6 -adenine cytokinins (e.g., benzyladenine, kinetin, zeatin, or isopentenyladenine) and extracts from various sources, cells in tissue culture, embryos, mature leaves, etc. Specific and exchangeable binding has been reported for proteins in the soluble fraction, particulate fraction including ribosomes, and thylakoid membranes from chloroplasts. Photoaffinity-labeled cytokinins have also been used. Several proteins have been purified, but either the affinities for natural cytokinins have been too low (K_D values usually more than 10^{-7} M) or the protein has been present in such large abundance that a case that one is dealing with a receptor protein cannot be made. For instance, a cytokinin-binding protein from wheat embryos is well characterized; its affinity for natural cytokinins is in the range of $\sim 10^{-7}$ M, but it occurs in large abundance ($\sim 10\%$ of soluble protein) and has amino acid sequence similarity to 7S globulin-type storage proteins.

A protein complex in tobacco with at least two subunits of 57 and 36 kDa is reported to bind cytokinins. The 57-kDa-binding protein is interesting because it is a homologue of *S*-adenosyl-L-homocysteine hydrolase (SAH hydrolase). As explained in Chapter 4, DNA methylation plays an important role in cell differentiation and in maintenance of the determined state; in contrast, loss of methylation is correlated with dedifferentiation and somatic embryogenesis. The intracellular source of methyl groups is *S*-adenosylmethionine (SAM). SAM provides methyl groups for the synthesis of ethylene as well as polyamines (see Chapter 11); it is also the source of methyl groups for methylation of DNA and proteins by methyl transferases. SAH hydrolase is a product of methyl transfer from SAM and, thus, is a competitive

inhibitor of SAM-dependent methyl transferase reactions on DNA and proteins and probably ethylene biosynthesis. Interestingly, SAH hydrolase activity is typically high in cells in culture and in root tips. Antisense inhibition of SAH hydrolase yields transgenics, half of which are stunted, probably because cell division is inhibited, and a three times higher CK content as judged from a bioassay of root exudate. Since cytokinins promote cell division, it is interesting that they should show binding affinity toward an enzyme that inhibits DNA methylation.

Many synthetic diphenyl urea derivatives, despite their vastly different structures from adenine-type cytokinins, show greater activity, on a molar basis, than adenine-type cytokinins. Hence, tritiated diphenyl urea derivatives have also been used to isolate cytokinin-binding proteins. A partially purified protein from mung bean seedlings had a K_D of $\sim 10^{-8} - 10^{-9}$ M for zeatin or benzyladenine. Free diphenyl-type molecules and the N^6 adenine cytokinins competed freely, but cytokinin ribosides did not (a difference between cytokinins and their ribosides, which is not seen in several other cytokinin-binding proteins).

Anticytokinins act as competitive inhibitors of cytokinin-induced responses, i.e., their effect can be overcome by higher concentrations of cytokinins (see Chapter 8). This suggests that they compete for the receptor site (or some other site early in signal transduction) and can be used to isolate the receptor protein or a nearby element in signal transduction. A fluorescent analog of a pyrolo [2,3-*d*]pyrimidine was used as a probe for cytokinin-binding proteins. Several candidate proteins from cytokinin-dependent tobacco tissue culture were reported. One protein had a reasonably high affinity for cytokinins (10^{-7} M), but was not characterized in detail.

In summary, several approaches have been used to isolate cytokinin-binding proteins. Several proteins have been purified or partially purified and show high-affinity, exchangeable binding to natural and synthetic cytokinins, but in no case has it been shown conclusively that the binding is to a cytokinin receptor.

3. CK-INDUCED GENE EXPRESSION

The list of genes induced or upregulated by cytokinins is long. It includes genes involved in photosynthesis, such as those encoding chl *a/b* protein (*CAB*), a small subunit of RUBISCO (*RBCS*); genes involved in anthocyanin biosynthesis, such as chalcone synthase (*CHS*) and dihydroflavonol reductase (*DFR*); genes

encoding cyclins, especially cyclin D3, cyclin-dependent kinases (CDKs), and ribosomal RNA, all involved in cell division; and genes encoding various metabolic enzymes, such as nitrate reductase (*NR*), phosphoenolpyruvate carboxylase (*PEPC*), hydroxypyruvate reductase, and the gene encoding the SAH hydrolase mentioned in the previous section. Other upregulated genes encode products that probably participate in metabolic partitioning across cell boundaries. These include an extracellular form of acid invertase, which hydrolyzes sucrose, a carrier protein for glucose transport across membranes, and several early nodulin (*ENOD*) genes, which are activated in roots of leguminous plants in response to nodulation factors secreted by nodulating bacteria, such as *Rhizobium*.

Some of these genes have been shown to be regulated by cytokinins at the transcriptional level (e.g., cyclin D3, *NR* genes), whereas for others, cytokinins have been reported to enhance mRNA stability (e.g., *CAB*, *RBCS* genes). Many of these genes are also induced by other factors, such as light, auxin, nitrate supply, wounding, and other stresses; and most are induced several hours, even days, after treatment with cytokinins. Thus, it is unclear whether they are induced primarily by cytokinins.

Using the differential display technique, two genes, called *INDUCED BY CYTOKININ* (*IBC6* and *IBC7*) have been identified in *Arabidopsis*. These genes are induced rapidly by benzyladenine (Fig. 24-20) and some urea cytokinins, which have strong activity in bioassays, e.g., thidiazuron (*N*-phenyl-*N'*-1,2,3-thidiazol-5-urea) and forchlorofenuron [*N*-(2-chloro-4-pyridyl)-*N*-phenylurea] (for structures, see Fig. 8-11 in Chapter 8). Curiously, zeatin and diHZ showed only about half as much induction as BA, and other adenine cytokinins, such as 2iP, or kinetin, showed much less induction. These genes were not induced by other factors, such as light, or other hormones, such as GA, ABA, or 2,4-D. Moreover, their induction did not require prior synthesis of another protein; hence, they are primary response genes.

Both *IBC6* and *IBC7* are single copy genes. The *IBC6* gene encodes a protein (M_r 20.1 kDa, *pI* 4.8) with sequence similarity to response regulators in the bacterial two-component systems, specifically to the CheY protein in *Escherichia coli*, which serves as the response regulator in the system for chemotaxis (for two component signaling systems, see Chapter 21). The three highly conserved residues of response regulators are all conserved in *IBC6*, including the putative phosphorylation site at Asp⁸⁷. The predicted sequence of *IBC7* protein is similar, but it has an acidic C-terminal extension found in many transcriptional activators.

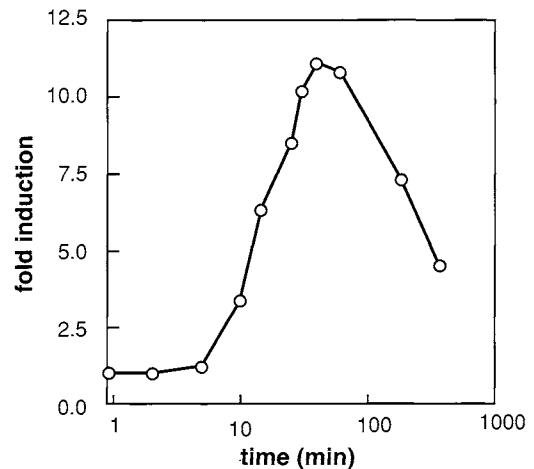


FIGURE 24-20 Kinetics of induction of the *IBC6* gene in *Arabidopsis*. Dark-grown seedlings were treated with benzyladenine (5 μ M). At indicated times, RNA was extracted and hybridized with *IBC6* cDNA probe. Maximum levels of *IBC6* mRNA were reached within 40 min; the levels then progressively declined to 6 h, although even at that time the levels were about four fold higher than basal levels. Modified from Brandstatter and Kieber (1998).

The precise functions of these proteins in cytokinin-induced responses are still unclear.

As yet, there is little information on either the response elements in promoters of cytokinin-induced genes or the transcription factors that bind to them.

4. CK RESPONSE MUTANTS

Cytokinin response mutants have been selected using several different screens.

4.1. Screen for Altered Morphology

Since cytokinins cause increased branching, loss of apical dominance, and inhibit root growth, these morphological criteria have been used to screen for mutants that are resistant or tolerant to high levels of cytokinins. The *cyr1* (for cytokinin resistant) mutant of *Arabidopsis* was selected for resistance of root growth to cytokinins. The mutant also displays incomplete cotyledon and leaf expansion, limited shoot development, reduced chlorophyll production, absence of anthocyanin, and an inability to develop mature, fertile flowers. The pleiotropic nature of this mutant suggests that *CYR1* may play a role in cytokinin signaling. The *stp1* (for stunted plant 1) mutant of *Arabidopsis* was isolated in a screen for decreased root growth under normal growth conditions and was later found to be cytokinin resistant. Cytokinin insensitivity is, how-

ever, restricted to root growth; thus, STP1 may have only a limited role in cytokinin signaling. The *zea3* (for zeatin) mutant of *Nicotiana plumbaginifolia* was selected for its ability to germinate on zeatin levels, which completely inhibit germination of wild-type plants. The effect of *zea3* mutation also seems to be restricted to germination. All of these are monogenic recessive mutations, but the wild-type genes have not been cloned and it is not clear whether they are linked directly to cytokinin perception or signaling.

Another mutant from *Arabidopsis*, *amp1* (for altered meristem program), is an overproducer of cytokinins and is considered elsewhere (see Chapter 8).

4.2. Screen for Cell Division and Growth

Since cytokinins promote cell divisions in cell/tissue culture, cytokinin autonomous mutants have been isolated by screening for cells/cell lines that are able to proliferate in cytokinin-depleted medium or in the absence of exogenous cytokinin. Several *cki* mutants (for cytokinin independent) from *Arabidopsis* were isolated after T-DNA activation tagging (for activation tagging, see Appendix 1). These mutants were able to grow in culture without exogenous cytokinins and form shoots. One mutant, *cki1-1*, went on to form whole plants, flower, and set seed. It carried a single T-DNA insertion and allowed the cloning of the gene.

The *CKI1* gene encodes a protein with an extracellular domain and an intracellular region, which combines the histidine kinase domain and the receiver domain with a conserved Asp, typical of the two-component signaling systems. It is similar to ETR1, one of the receptors for ethylene (see Chapter 21), although CKI1 has two membrane-spanning domains, whereas ETR1 has three such domains (Fig. 24-21). CKI1 has been postulated as a cytokinin receptor because overexpression of the CKI1 gene in transgenic plants results in typical cytokinin-type responses. Cytokinin-binding studies using CKI1 protein, such as those for ETR1 expressed in yeast cells, have not been performed, and the precise role of CKI1 in cytokinin signaling is still unknown.

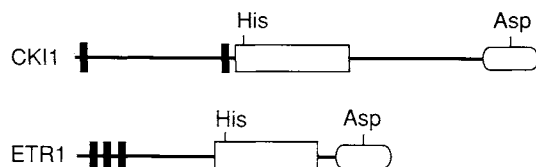


FIGURE 24-21 Structure of CKI1 in *Arabidopsis* compared with that of ETR1. Reprinted with permission from Kakimoto (1996), © American Association for the Advancement of Science.

Another cytokinin response mutant, *cre1* (for cytokinin response 1), has been isolated in a screen using lack of cell proliferation and shoot histogenesis in tissue culture in response to added cytokinins. Mutant plants are also much less sensitive to inhibition of root elongation by cytokinins. The wild-type gene *CRE1* encodes a protein that is predicted to have an N-terminal, extracellular region flanked by two transmembrane segments and an intracellular region with a histidine kinase domain and a response regulator domain at the C terminus. The gene is identical to *WOL1* and to *AHK4* in *Arabidopsis*, which have been shown to regulate cell proliferation and proper formation of vascular tissues in roots (see Chapter 3) and to serve as the cytokinin receptor in roots, respectively. Transgenic expression of *CRE1* in the mutants *cre1-1* and *cre1-2* restored their responsiveness to added cytokinins in tissue culture. Yeast strains lacking a functional SLN1 protein involved in osmosensing (see Box 21-1, Chapter 21) are lethal. Expression of *CRE1* in the *sln1Δ* mutant by itself did not suppress lethality, but the addition of cytokinins, such as *trans*-zeatin, to the medium restored the mutant cells to normal growth, thus providing a direct link between cytokinin perception by CRE1 and a biological response. Together these data provide convincing proof that CRE1 is a cytokinin receptor and that plant cells probably perceive cytokinins at the cell surface and cytokinin signaling involves a phosphorelay. These results are striking for another reason—they show that yeast cells respond to *trans*-zeatin, one of the few examples of a plant hormone eliciting a response in a different kingdom. Significantly, *cis*-zeatin was ineffective in this assay.

4.3. Screen for Ethylene Synthesis in Dark-Grown Seedlings

Cytokinins induce the synthesis of ethylene in dark-grown seedlings by activating ACC synthase, the key regulatory enzyme in ethylene biosynthesis. In *Arabidopsis*, five ACC synthase genes (*ACS1*–*ACS5*) have been identified, three of which encode functional enzymes and are differentially induced by different signals, including auxin and wounding (see Chapter 11). Low levels of cytokinins (e.g., 0.5 μ M benzyladenine or less) enhance the stability of *ACS5* mRNA in dark-grown seedlings, and ethylene produced is sufficient in amount to cause the triple response (see Chapter 21).

The induction of triple response occurs within 3 days and, thus, provides a rapid screen for sensitivity or insensitivity to applied cytokinin. *Arabidopsis* seeds were mutagenized, germinated in the

presence of $0.5\ \mu\text{M}$ benzyladenine, and screened for lack of triple response. The screen yielded several mutants that were either defective in ethylene production or ethylene insensitive. It also yielded some mutants that were insensitive to applied cytokinins but not to ethylene. These latter mutants, *cin1*–*cin4* (for cytokinin insensitive), show the normal triple response to ethylene (Fig. 24-22). Moreover, they produce low levels of ethylene at low concentrations, but normal levels of ethylene at high concentrations of applied cytokinins, indicating clearly that different isoforms of ACC synthase are involved in ethylene production in the dark and that low levels of cytokinins affect only the expression of the *ACS5* gene.

cin mutations may prove to be important in the identification of elements necessary for cytokinin signaling to *ACS5*.

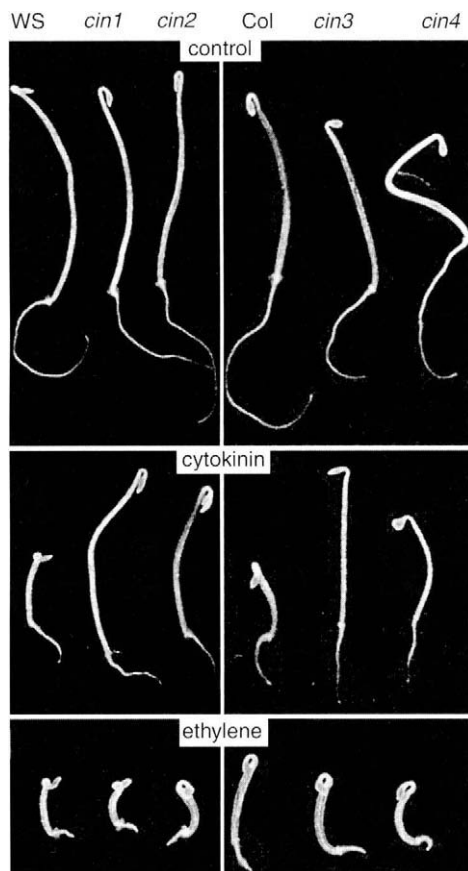


FIGURE 24-22 Cytokinin-insensitive mutants of *Arabidopsis*. The mutants *cin1* and *cin2* (in the wild ecotype WS background) and *cin3* and *cin4* (in Col background) in air, after cytokinin, and after ethylene treatment. Note that after cytokinin treatment, WT plants show a triple response, whereas mutants do not. In ethylene, all plants show the triple response. From Vogel *et al.* (1998).

5. SECTION SUMMARY

Cytokinin perception and signaling are still very much a mystery, although some important advances have been made recently. Despite much effort, little is known about cytokinin-binding proteins and cytokinin-induced gene expression. There is still no information on cytokinin response elements or on transcription factors that bind to them. This picture may change with the identification of two genes in *Arabidopsis*, *IBC6* and *IBC7*, which are induced within minutes of benzyladenine treatment and which are primary response genes and encode proteins similar to bacterial response regulators. Several response mutants, *cyr1*, *cin1*, and *cin2* in *Arabidopsis*, have been characterized, but the wild-type genes have not been cloned and it is not known what part they play in cytokinin signaling. Despite these handicaps, progress is being made in the identification of cytokinin receptors. Two genes in *Arabidopsis*, *CKI1* and *CRE1*, encode proteins that suggest that CK perception and signaling proceed via a two-component signaling system. Both proteins are similar to ETR1, an ethylene receptor, and combine features of a sensor protein and a response regulator. While the role of *CKI1* is still unclear, evidence for *CRE1* being a cytokinin receptor is strong. Mutations in the *CRE1* gene render plants or plant tissues nonresponsive to cytokinins, and expression of *CRE1* in *cre1* mutants reestores their responsiveness. Moreover, expression of *CRE1* in a lethal mutant of yeast defective in osmosensing restores the mutant cells to normal growth if cytokinin is added to the medium. These data indicate that cytokinin perception in plant cells occurs extracellularly and that signaling proceeds via a phosphorelay. With the identification of *CRE1* as a putative cytokinin receptor in *Arabidopsis*, an important breakthrough has been made. Future studies will undoubtedly focus on identifying elements in the signaling pathway, including relationships, if any, between *CRE1* and proteins, such as *CKI1*, *IBC6*, and *IBC*, and on identification of proteins similar to *CRE1* in other plants.

SECTION III. MOLECULAR BIOLOGY OF BRASSINOSTEROID (BR) ACTION

1. BR-INDUCED RESPONSES

Brassinosteroids play important and unique roles in extension growth of stems or petioles in dicot seedlings and in grass leaves. In dark-grown seedlings,

exogenous application of BRs has little apparent effect, but BR synthesis mutants clearly show that BRs are needed for normal etiolated growth in the dark. These mutants display a partial light-grown habit, which indicates that BRs repress a precocious expression of photomorphogenesis in dark-grown seedlings or in seeds germinating under soil. BRs inhibit root growth. They also seem to have a role in flowering, pollen tube growth, tracheary cell differentiation, senescence, and expression of some stress-related genes.

2. BR-INDUCED GENE EXPRESSION

Brassinosteroids have come to be recognized as hormones in their own right only since the early 1990s. Published studies on binding of radiolabeled BRs to cell fractions and/or on brassinosteroid-induced gene expression are very few. A gene called *BRU1* (for brassinosteroid upregulated) was isolated from soybean (*Glycine max*) seedlings. The encoded protein shows sequence similarity to xyloglucan endotransglycosylases from other plant species (e.g., *Arabidopsis*), and the expressed protein shows XET activity *in vitro*. Gene encoding a putative β -tubulin subunit and an effector involved in actin polymerization are also reported to be induced. However, the *cis* sequences and transcription factors regulating its expression are unknown.

3. BR RESPONSE MUTANTS

Several screens have been used to isolate brassinosteroid-insensitive mutants. All mutants identified to date are from *Arabidopsis*. Since brassinosteroids inhibit root growth, stimulate stem elongation in light, and suppress the expression of the photomorphogenic program in dark-grown seedlings, these features can be used to screen for BR-insensitive mutants. One mutant, *bri1* (for brassinosteroid-insensitive), was identified by germinating EMS-mutagenized seeds in media supplemented with exogenous 24-epibrassinolide (10^{-6} or 10^{-7} M) and screening for seedlings for lack of inhibition of root growth. Three *cbb* (for cabbage morphology) mutants were isolated by screening for the dwarf phenotype from a mutant collection generated by insertional mutagenesis. While two of the *cbb* mutants proved to be brassinolide synthesis mutants, one mutant, *cbb2*, could not be rescued by exogenous brassinolide and turned out to be a brassinosteroid-insensitive mutant. Still other mutants,

called *bin* (for brassinosteroid-insensitive), were identified by screening mutagenized seedlings grown in dark for expression of photomorphogenic program, i.e., a phenotype similar to that of *det*, *cop* mutants (see Chapter 26). Subsequently, a secondary screen separated the mutants isolated into those that could be rescued by applied brassinosteroids from those that could not be so rescued. The *bin* mutants, 18 in number, all were found to be alleles of the same gene, called *bin1*. Moreover, *bin1* turned out to be an allele of *bri1*, which is also allelic to *cbb2*. In short, using three different mutant screens, only a single brassinosteroid signaling gene has been identified. Following the rules of nomenclature, the gene is *BRI1* (or *CBB2*), with 20 mutant alleles.

bri1 is a recessive mutation with pleiotropic effects, which phenotypically resembles BR-deficient mutants, except that it cannot be rescued by an exogenous supply of brassinolide. Grown in darkness, mutant plants show short, thick hypocotyls, open, expanded cotyledons, development of primary leaf buds, and accumulation of anthocyanins. Grown in light the mutant is an extreme dwarf, less than one-tenth the height of the wild type (Fig. 24-23A). It has smaller, darker green leaves than the wild type, shows reduced apical dominance and enhanced lateral branching, and reduced male fertility. It also exhibits a delay in flowering and senescence. The *bri1* mutation is specific to brassinosteroid signaling because the mutant plants retain their sensitivities to other hormones, IAA, cytokinins (benzyladenine and kinetin), GA₃, and ABA (Fig. 24-23B).

The *lka* mutant in pea is another BR-insensitive mutant. It is a dwarf and, in contrast to *lkb*, which is a BR synthesis mutant, is not rescued by application of brassinolide or castasterone (Fig. 24-24). Like some GA-insensitive mutants, both *bri1* and *lka* accumulate higher levels of endogenous BRs than the wild type, probably due to lack of feedback control over their synthesis.

3.1. Cloning and Sequencing of BRI1

The *BRI1* gene encodes a large ($M_r \sim 130$ kDa) receptor-like kinase (RLK) (see Chapter 25). These kinases have an extracellular domain, a transmembrane domain, and an intracellular domain, which serves as a serine/threonine protein kinase. RLKs vary in their extracellular domains; some have several tandem copies of an amino acid sequence rich in leucine, known as leucine-rich repeats (LRRs). *BRI1* is somewhat unique in that the extracellular domain not only shows an extensive LRR repeat region, but also a 70 amino acid island between two LRRs; it also

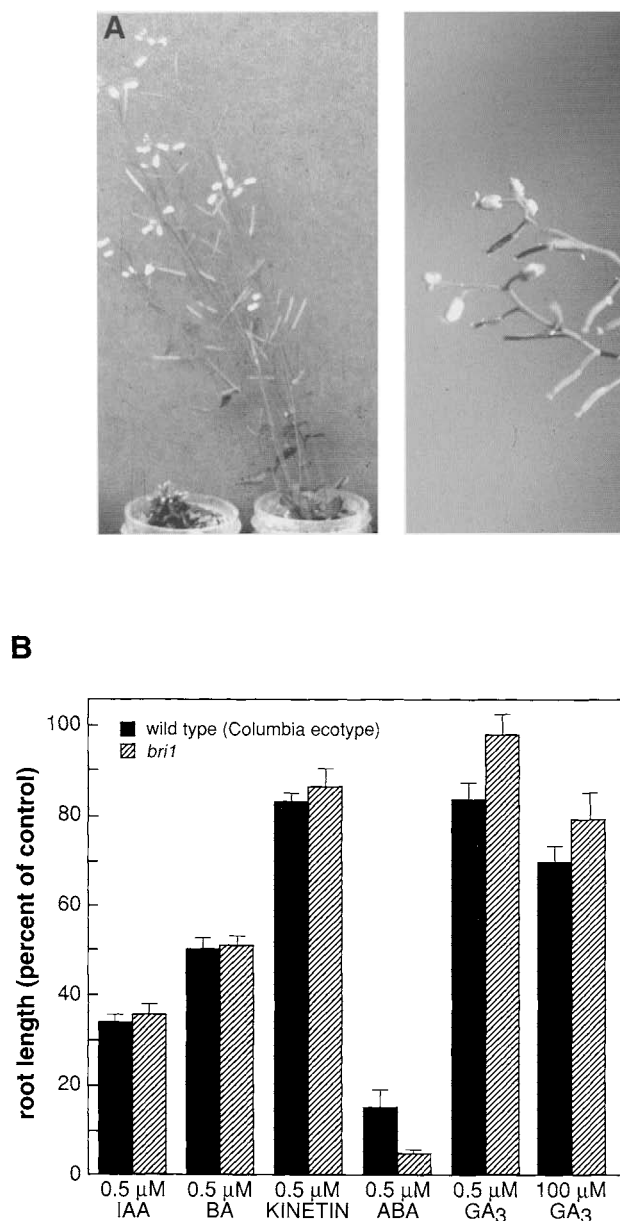


FIGURE 24-23 The *bri1* mutant of *Arabidopsis* and the effect of various hormones on root growth. (A) Phenotypes of a 2-month-old *bri1* mutant (left) and a wild-type plant (middle). Also shown is a 4-month-old mutant plant (right). The mutant is an extreme dwarf with small dark green leaves and enhanced lateral branching. Sacle, the fruits (siliques) in the wild type plant are ~1 cm long. (B) Comparison of effects of various hormones on root growth in the wild type and the *bri1* mutant. Plants were treated with IAA, benzyladenine (BA), kinetin, ABA, and GA₃, all at 0.5 μ M; and GA₃ also at 100 μ M. Root length is plotted as percent of control, that is, root length in wild-type plants without any hormonal treatment. Note that there is little difference between the wild type and the mutant in their responses to IAA and cytokinins; there is a little more inhibition in the mutant by ABA, and a little less by GA. From Clouse *et al.* (1996).

shows a leucine zipper motif (Fig. 24-25). The leucine zipper motif suggests that the protein forms homo- or

heterodimers; the LRR repeat motif is believed to be involved in protein-protein interactions; and the intracellular kinase domain is thought to play a role in signal transduction.

BRI1 mRNA is expressed ubiquitously and constitutively in light- or dark-grown seedlings and throughout plant development. Several facts suggest that *BRI1* plays a role in BR signaling and that it is a receptor kinase. All 20 mutant alleles, using three different screening strategies, are mutations in a single gene, which suggests that *BRI1* is a component in the BR-signaling pathway. Second, the 70 amino acid island between two leucine-rich repeats in the extracellular region is unique for LRRs, and mutations in this island disrupt the functioning of *BRI1*. Third, *in vitro* binding studies have shown that the extracellular domain of



FIGURE 24-24 The *lka* mutant of pea (*Pisum sativum*). The *lka* plant (left, control) and 3 days after the application of 100 ng of brassinolide to the third leaf (right). From Nomura *et al.* (1997).

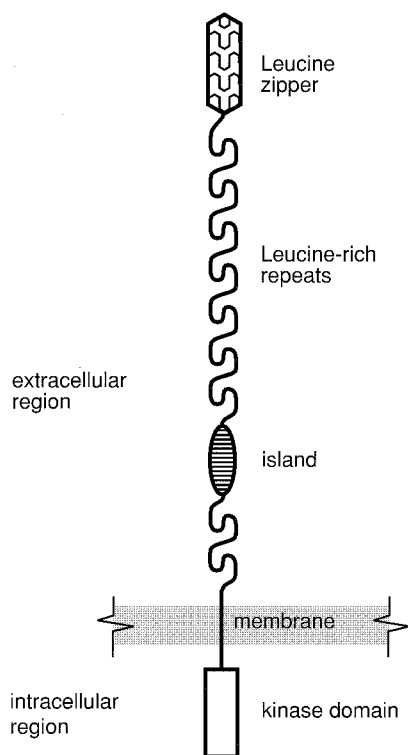


FIGURE 24-25 Schematic structure of BRI1 in *Arabidopsis*. BRI1 has an extracellular domain, which consists of a leucine zipper motif, followed by 25 tandem leucine-rich repeats (LRRs), with a 70 amino acid island between LRR 21 and 22. The extracellular domain is followed by a transmembrane span and finally by an intracellular serine/threonine kinase domain. Based on data in Li and Chory (1997).

BRI1 binds brassinolide specifically, and the number of binding sites is proportional to the amount of BRI1 protein. Moreover, treatment of *Arabidopsis* seedlings with brassinolide induces autophosphorylation of BRI1. Fourth and finally, the extracellular and membrane-spanning domain has been shown to initiate a signaling cascade which brings about a biological response. This domain was fused to the serine-threonine kinase domain of a receptor kinase known to be involved in plant defence reactions, and the construct was used to transform rice cell lines. The chimeric receptor initiated defence responses in rice cells upon treatment with brassinolide.

The above data provide convincing proof that BRI1 is a receptor kinase that transduces steroid signals across the plasma membrane. However, there are still some gaps in our knowledge. It is not known whether BRI1 occurs as part of a receptor complex and, if so, the identity of the interacting partners. The downstream signaling components are also unknown.

3.2. BR Interaction with Other Hormones

A new mutant, *sax1* (for hypersensitive to abscisic acid and to auxin), of *Arabidopsis* was isolated in a screen for inhibition of root growth at auxin concentrations that are not normally inhibitory (~ 10 nM NAA). The mutant later proved to be hypersensitive to ABA as well in root growth and stomatal closure (although not in other ABA-specific responses). The mutant also does not show GA-induced hypocotyl elongation growth in light, a significant difference from the other BR synthesis mutants (Fig. 24-26). It also seems insensitive to ethylene, as shown by a lack of response to added ACC. The addition of 24-epibrassinolide (10^{-9} M in root; 10^{-7} M in hypocotyl) to the growth medium restores the wild-type sensitivity in root growth to IAA and ABA and for hypocotyl growth to GA₃. However, the sensitivity to ethylene is not restored by epi-BL. The *SAX1* locus has been identified by feeding intermediates and rescue experiments and appears to be a new locus in an alternate pathway of BR biosynthesis between campesterol and 6-deoxocathasterone (for the synthetic pathway, see Fig. 9-5 in Chapter 9). These results are interesting because they provide a link between BRs and auxin, ABA, GA, and ethylene signaling pathways. Since BRs restore a wild-type sensitivity to auxin, ABA, and GA₃ in the growth of *sax1* seedlings, it appears that three different signaling pathways controlling cell elongation may be regulated by a fourth pathway involving BRs.

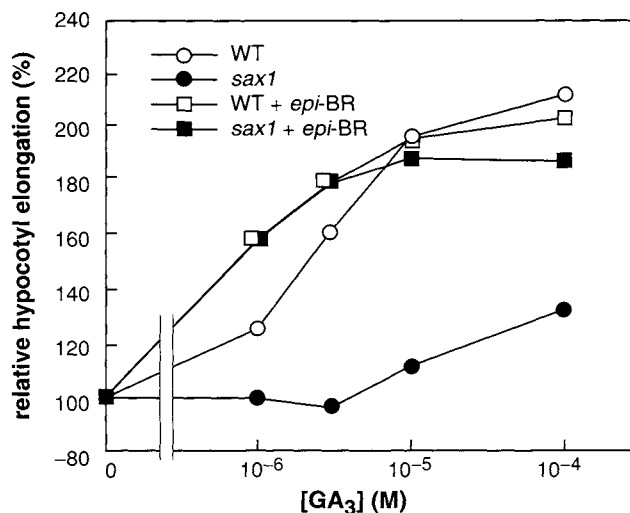


FIGURE 24-26 Hypocotyl growth in GA₃-treated wild type *Arabidopsis* and *sax1* seedlings in light. Inhibition of growth in *sax1* seedlings is restored by the addition of epibrassinolide. From Ephritikhine *et al.* (1999).

3.3. BR Interaction with Light

Brassinosteroids are involved in the maintenance of the etiolated habit in dark-grown seedlings and also interact with red and blue light receptors. These topics are covered elsewhere (see Chapter 26, Section IV, 1.3).

4. SECTION SUMMARY

In summary, relatively few genes have been shown so far to be transcriptionally induced by BRs, and *cis*-sequences and transcription factors involved in BR-induced gene expression are unknown. But our information on BR signaling has taken a quantum leap forward with the cloning and characterization of the *BRI1* gene. The *BRI1* gene encodes a transmembrane receptor kinase which binds brassinolide in its extracellular domain. Such binding results in autophosphorylation of the intracellular domain and likely sets up a signaling cascade which involves phosphogroup transfer via the histidine kinase domain. The receptor function of *BRI1* has been demonstrated, but it is not known whether it is part of a multimeric complex and what its downstream signaling components might be. The discovery of a novel BR synthesis mutant, *sax1*, indicates that BRs are involved in cross talk with signaling by auxins, ABA, and GA, but the nature and sites of such cross talk are obscure. BR signaling plays a role in maintenance of the etiolated phenotype in dark-grown seedlings; it also interacts with signaling by photoreceptors, such as phytochrome.

SECTION IV. MOLECULAR BIOLOGY OF JASMONATE (JA) ACTION

1. ROLES OF JAS

Jasmonates play important roles in plant defenses against wounding caused by physical injury or chewing by insects, attack by microbial pathogens, and some abiotic stresses such as exposure to ozone. They are involved in the synthesis of vegetative storage proteins (VSPs), coiling of tendrils, fruit ripening, and acceleration of senescence and abscission of plant parts such as leaves. They are also reported to play a role in the production of viable pollen and in the formation of tubers, bulbs, and storage roots. Among the responses, just given, the role of jasmonates in

defense against wounding and microbial attack and the synthesis of VSPs have been the most studied, and most data on jasmonate-induced gene expression and signaling come from these sources.

2. DEFENSE-RELATED SIGNALING

Since one of the major causes for jasmonate production is wounding or exposure to microbial elicitors, plant defense responses to pests and pathogens involving jasmonates are in fact a two-stage process. In the first stage, plants perceive the signal of wounding or microbial challenge and start a signaling cascade, which results in the synthesis of jasmonates via the octadecanoid pathway. In the second stage, jasmonates trigger the activation of defense-related genes (see Fig. 12-3 in Chapter 12). The perception of the wound signal or microbial elicitors in the first stage is still a mystery, but the subsequent events, i.e., the activation of prosystemin gene, cleavage and transport of systemin, its perception at the surface of target cells, and release of linolenic acid from cell membranes, are much better understood, mostly due to the efforts of Clarence Ryan at Washington State University, Pullman, WA, and associates. The second stage involves the perception of jasmonates by their receptor and start of the second signaling cascade, which ends in the transcription of defense-related genes. This part is mostly unknown, but a few pieces in the puzzle seem to be falling in place. This chapter is mostly concerned with the second stage, which deals with jasmonate signaling. For the first stage, which includes systemin signaling, the interested reader is referred to the reference section.

It should be pointed out that transmission of the wound or elicitor signal to distant sites in the first stage proceeds extremely rapidly. Chapter 25 describes signaling events from cell surface receptors to gene expression and the roles of secondary messengers and protein kinases and phosphatases in signal transduction. It is sufficient to say here that a mitogen-activated protein kinase in *Arabidopsis*, called WIPK (wound-induced protein kinase), is induced within minutes of wounding and has been proposed to activate the enzymes involved in jasmonic acid biosynthesis. The perception of systemin by its receptor on the surface of target cells is also reported to start a signaling cascade, which includes ion transport, and an increase in intracellular Ca^{2+} and Ca^{2+} /calmodulin-activated protein kinases.

3. JA-INDUCED GENE EXPRESSION

There are no hormone-binding studies using radiolabeled jasmonates. Expression of many genes, however, is known to be affected by jasmonates. Most of these genes encode defense-related proteins (see below); others encode enzymes involved in the synthesis of jasmonate or related compounds in the octadecanoid pathway (e.g., lipoxygenase, allene oxide synthase, and hydroperoxy lyase). Still others encode vegetative storage proteins (see Chapters 12 and 20). The senescence-related breakdown of chlorophyll involves removal of the phytol chain from the porphyrin nucleus by an enzyme chlorophyllase (see Chapter 20). In *Arabidopsis*, two genes encoding chlorophyllase, *AtCHL1* and *AtCHL2*, have been cloned and are differentially activated. Treatment with MeJA activates the transcription of *AtCHL1*, but not that of *AtCHL2*, which is constitutively expressed at low levels.

ABA and jasmonates share some responses. Both can inhibit growth, inhibit seed germination, and promote senescence. Both are also known to induce some genes encoding seed storage proteins, as well as an oil body protein (oleosin) in *Brassica napus*. However, many ABA-regulated genes are not affected by JA (e.g., *LEA* genes). The common ice plant (*Mesembryanthemum crystallinum*), a succulent, shows Crassulacean acid metabolism (CAM) and expresses one specific isoform of phosphoenolpyruvate carboxylase. Induction of the gene encoding this isoform (*Ppc1*) is regulated by ABA, not by JA.

The plant response to biotic stresses involves complex interactions between a variety of signals. For example, defense responses to attack by pests and pathogens are mediated *via* ABA, ethylene, and jasmonate. Responses to pathogens are also mediated *via* salicylic acid. To separate which genes are regulated by which hormone, use is made of synthesis inhibitors and/or synthesis mutants for specific hormones. A comprehensive study on regulation of the leucine aminopeptidase (*LapA*) gene in tomato (*Lycopersicon esculentum*) by jasmonates, ABA, and salicylic acid utilized some of these techniques. It was shown that *LapA* is induced by jasmonates and ABA (although not by water stress or salinity) and that the two hormones act synergistically. The induction of proteinase inhibitor 2 (*pin2*) gene, in contrast, required jasmonate but not exogenous ABA. The *Le4* gene in tomato encodes a dehydrin; its expression is upregulated by ABA, water deficit, and salinity, but not JA. Finally, the genes for two pathogenesis-related proteins in tomato, PR-1 (pathogenesis-related 1) and GluB (basic β -1, 3-glu-

canase), were induced by jasmonate independently of systemin or wounding; also, their induction did not require exogenous ABA.

Among the PR genes in *Arabidopsis*, some are regulated by salicylic acid independently of JA, whereas others are regulated by JA independently of salicylic acid. Moreover, the two pathways act antagonistically to each other. *Arabidopsis* plants infected with the fungus *Alternaria brassicicola* or healthy plants treated with exogenous MeJA, but not salicylic acid, show the induction of the *PDF1.2* gene, which encodes an antifungal peptide belonging to the family of plant defensins. However, *PDF1.2* expression is not under the exclusive control of jasmonates; it requires the simultaneous availability of ethylene. In mutants that are defective in the synthesis of jasmonates or the response pathway of either hormone the gene is not expressed. Many chitinases are induced by ethylene or jasmonates independently. For example, the *BjCHI* (for *Brassica juncea* chitinase) gene is induced by wounding and by JA, but not by ABA, ethylene, or salicylic acid.

There is also evidence for antagonism between ABA and JA signaling. For instance, JA-induced accumulation of PR proteins in rice (*Oryza sativa*) roots is negatively regulated by ABA, whereas JA negatively affects ABA-induced transcript accumulation of the *LEA3* gene in rice (*OsLEA3*).

In summary, defense-related genes are regulated by a variety of signals, some genes are regulated exclusively by one signal (e.g., JA induction of some wound-specific *PIN* genes) and others by more than one signal (e.g., *PDF1.2* gene). These results highlight the complexities of plant responses to biotic stresses and indicate that there must be extensive cross talk among signaling pathways of jasmonates, ABA, ethylene, and salicylic acid. As explained in Chapter 23, the responses to abiotic stresses (e.g., water deficit, salinity) are mediated separately and jointly by the stress and by ABA. Jasmonate-induced gene expression also occurs in responses to abiotic stresses, such as exposure to salinity or ozone, but the details of such gene regulation are unknown.

Although several genes are now known to be induced by jasmonates, the *cis* elements in promoters of these genes and transcription factors that bind to them are mostly known. A G-box sequence (CACGTG) has been identified in the promoters of the *Pin2* gene in tomato and the *VSP β* gene in soybean, but it is unclear whether it is able to confer induction by jasmonates. For the *VSP* gene in soybean, other *cis* elements responsive to nutritional factors, such as carbon or phosphorus availability, seem to act in concert with the G box. Transcription factors binding to *cis* elements in

the promoters have not been identified. Considering that some promoters have G boxes, it is possible that bZIP proteins bind to these sequences.

4. JA RESPONSE MUTANTS

Four jasmonate-insensitive mutants are known from *Arabidopsis*. Three of them, *jar1* (for jasmonate resistant), *jin1*, and *jin4* (for jasmonate-insensitive), were isolated from mutagenized seeds grown in the presence of concentrations of jasmonic acid (~10 mM) that inhibit root growth. A fourth mutant, *coi1* (for coronatine insensitive), was identified by its resistance to coronatine, a chlorosis-inducing bacterial toxin from *Pseudomonas syringae*, which is similar in structure and activity to jasmonic acid. Both coronatine and JA inhibit root growth, cause anthocyanin production, and stimulate the expression of a gene encoding a VSP.

All four mutants are single gene recessives and respond to exogenous ethylene and ABA like the wild-type plants; hence, they are not mutants in either ethylene or ABA response pathways, but they differ in the range of their insensitivities to exogenous jasmonates. *jin1* and *jin4* show insensitivity to jasmonates in root growth, but produce fertile flowers and accumulate JA-induced VSPs in floral structures. The *jar1* also fails to accumulate two VSPs similar to VSP α/β in soybean when sprayed with 50 μ M MeJA.

The *coi1* mutant shows the most pleiotropic effects. It is phenotypically similar to the jasmonate-deficient triple *fad* (fatty acid desaturase) mutant (*fad* 3-2 *fad* 7-2 *fad* 8) of *Arabidopsis*. The triple *fad* mutant is unable to synthesize linolenic acid, the precursor fatty acid for jasmonate biosynthesis (see Chapter 12). The mutant has little defense against herbivorous insects; it also produces nonviable pollen. However, both protection against insect pest and pollen sterility are rescued by an exogenous application of jasmonates. The *coi1* mutant also shows a decreased resistance to insect attack, a reduced response to wound damage, and produces nonviable pollen, but the defects are not cured by exogenous jasmonates. Hence, the *COI1* gene is believed to function in the JA signaling pathway. Moreover, the pleiotropic nature of the *coi1* mutation suggests that it may be defective in an early step in jasmonate perception or signaling.

The *COI1* gene was cloned using a map-based strategy. The encoded COI1 is a cytosolic protein because it lacks any domains, suggesting a signal peptide or membrane-spanning domains. It shows two significant motifs, a F box and 16 leucine-rich repeats (LRRs). Both LRR and F box occur in some proteins

in yeast and mammals and are involved in protein-protein interactions and recruitment of targeted proteins for polyubiquitination and eventual proteolysis. A similar protein, TIR1, is known from *Arabidopsis* and has been proposed to recruit repressors of auxin signaling for proteolytic degradation (see Fig. 22-15, Chapter 22). Occurrence of the F box and LRR motifs in two proteins, which on genetic evidence are involved in jasmonate or auxin signaling, suggests that selective ubiquitination of repressor proteins may be a more common theme in plant signaling than hitherto realized.

The target proteins for recruitment by COI1 or other components with which COI1 interacts are unknown.

Recently, a loss-of-function recessive mutation, *cev1* (for constitutive expression of VSP1) was obtained by targeted genetics. The promoter of the JA-induced *VSP1* gene of *Arabidopsis* was fused to the coding sequence of *LUCIFERASE* reporter gene, and the construct used to transform *Arabidopsis* seedlings. The progeny seeds were mutagenized, and the mutant plants screened for luciferase activity without treatment with exogenous methyl jasmonate. The *cev1* seedlings show a constitutive expression of both JA-induced and ethylene-induced phenotypes. The plants were stunted, had short roots, an excess of root hairs (phenotypes typical of ethylene-treated seedlings); they also accumulated anthocyanins and expressed JA-induced *VSP1* and *VSP2* genes constitutively. They also showed a constitutive expression of two genes, *PDF1-2* and *CHITINASE-B*, which are regulated by both JA and ethylene. CEV1, therefore, seems to function at an early step in both signaling pathways.

5. SECTION SUMMARY

Although jasmonates are clearly involved in several morphogenetic as well as defense responses in plants, there is still very little known about the molecular basis of their action. Jasmonates induce the expression of several genes, some independently and some in combination with either ABA or ethylene. Some genes induced by jasmonates are inhibited by ABA or salicylic acid. The existence of separate (or parallel) and cross signaling pathways among four hormones, jasmonates, ABA, ethylene, and salicylic acid, and possibly some abiotic factors, such as salinity or water stress, makes the elucidation of jasmonate signaling difficult and challenging. Some *cis* elements responsive to JA have been reported, but the details of regulation, including transcription factors that bind to them, are unknown. A pleiotropic mutant, *coi1*, that

shows insensitivity to jasmonates in several responses has been described. The *COI1* gene encodes a protein that seems to be involved in the recruitment of repressor proteins targeted for proteolysis. A similar protein is also known for auxin signaling. The identity of proteins that repress jasmonate signaling is unknown; also unknown are the proteins that interact with *COI1*. Another mutant, *cev1*, defines a locus that is common to both JA and ethylene signaling.

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SECTION I. MOLECULAR BIOLOGY OF GIBBERELLIN (GA) ACTION

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See Chapter 26.

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SECTION III. MOLECULAR BIOLOGY OF BRASSINOSTEROID (BR) ACTION

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1. PLANTS ARE SUBJECTED TO A WEALTH OF SIGNALS

Plants, being rooted, are bombarded by a wealth of external stimuli. They are also subject to many endogenous signals, such as hormones and developmental cues, some of which emanate from neighboring cells and involve intercellular communication. All these signals must be recognized, integrated, and translated into responses at the cell or organ level. The mechanisms of signal perception and many components of signaling pathways are highly conserved among eukaryotes and prokaryotes, although plants, being sessile, have evolved along different lines from animals and show some unique features and novel combinations of ancient themes. This chapter deals with the general paradigms of signal perception and transduction in eukaryotic systems and the unique features of signaling in plants.

2. SIGNAL TRANSDUCTION PARADIGM

Studies on model systems, such as *Caenorhabditis*, *Drosophila*, *Xenopus* oocytes, mammalian cell lines, and yeast, since the mid-1980s have resulted in formulation of a signaling paradigm, the basic elements of which are remarkably conserved. The paradigm is shown in Fig. 25-1. An extracellular signal binds to or stimulates a plasma membrane-based receptor, which activates a GTP-binding protein (G-protein). The G-protein alternates between two conformational states—GDP- or GTP-bound states—and thus acts as a molecular switch: on when bound to GTP and off when bound to GDP. GTP is hydrolyzed to GDP by an intrinsic GTPase activity in the protein, but, while activated, the G-protein either directly regulates a cascade of protein kinases or modulates the activity of effector molecules and second messengers, which, in turn, regulate the activities of protein kinases. Protein kinases regulate the activities of other proteins and/or enzymes, which mediate gene expression or metabolic pathways. Protein phosphatases are also recognized as important components of signaling.

The signal perception/transduction pathways, thus, involve at least four elements: receptors, G-proteins, effector molecules and second messengers, and protein kinases and phosphatases (1–4 in Fig. 25-1). They are multiple step pathways and often are quite long. It stands to reason that if a particular signaling pathway is known, it may be possible to obtain the end response, say gene expression, by activating a down-

stream element, such as 2, 3, or 4, without involving the receptor.

There are two main advantages to having multistep signaling pathways: (i) multiple steps allow the possibility for the original signal to be amplified or attenuated and (ii) they also allow the opportunity for cross talk between different signaling pathways, which may result in a synergistic, attenuated, or an otherwise modified response (Fig. 25-2).

Many intercellular messengers in animals are hydrophilic and are unable to cross the plasma membrane to reach their intracellular targets. Hence, there is a need for transmembrane receptors that recognize the signal extracellularly and, *via* some conformational change, transfer the signal to an intracellular component as outlined above. A very important exception to the paradigm is provided by another class of receptors, the intracellular receptors, such as those for steroid hormones. Steroids, unlike many other signaling molecules, are lipophilic and are able to get across the plasma membrane. Their receptors occur in the cytoplasm and nucleus and interact directly with the hormone and bind to DNA (see soluble receptor, Fig. 25-1). Their structure is well characterized. They show at least four domains: a variable region that binds the ligand, a hinge region, a conserved domain that binds to DNA, and a not so conserved activation domain that is involved in activating transcription of the cognate gene. In comparison to plasma membrane-based receptors, steroidal signaling is economical and involves much fewer steps.

Despite much anticipation, so far, there is little evidence for steroid-type signaling in plants. Hence, the rest of this chapter deals with signaling by plasma membrane-based receptors.

The best characterized plasma membrane-based receptors are of two kinds.

i. **Transmembrane receptor enzymes.** These receptors combine the functions of a receptor and an enzyme, usually a kinase, in the same polypeptide. The signal receiving domain is extracellular, which is connected to an intracellular kinase domain by a single membrane-spanning domain (Fig. 25-3A). The kinase domain interacts with a monomeric, small G-protein, which initiates a protein kinase cascade.

ii. **G-protein-coupled receptors (GPCRs).** These receptors have an extracellular domain that receives the signal, followed typically by seven membrane-spanning domains and an intracellular domain but no kinase activity. They activate a G-protein, in this case part of a heterotrimeric complex, which modulates the activities of a series of protein kinases/phosphatases *via* effectors and secondary messengers (Fig. 25-3B).

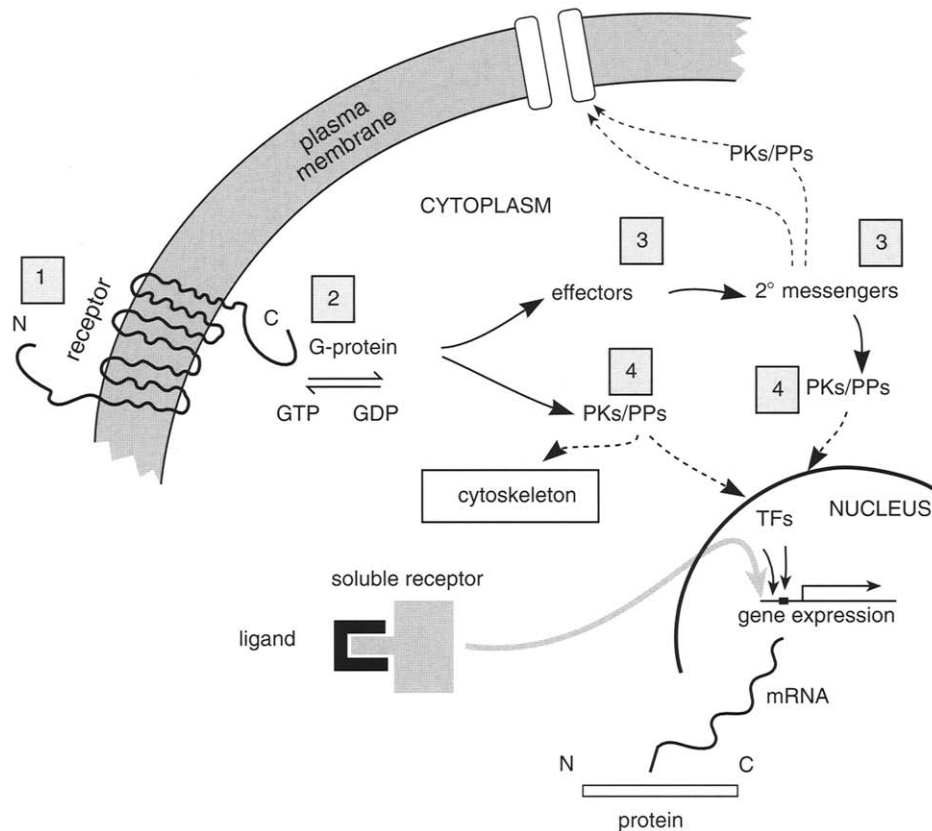


FIGURE 25-1 General scheme of signal perception and transduction. (A) A plasma membrane-based receptor (1) binds a ligand extracellularly and interacts with a GTP-binding protein (2) intracellularly. The G-protein either directly or indirectly, via second messengers (3), activates one or more protein kinases and/or phosphatases (4), which ultimately results in a response. G-protein, GTP-binding protein; PK, protein kinase; PP, protein phosphatase; TF, transcription factor.

Alternative signaling by the two types of receptors also occurs. For example, transmembrane receptor kinases may activate enzymes involved in the generation of secondary messengers; likewise, heterotrimeric G-proteins may activate a kinase cascade directly.

In the following sections, GPCRs and signal transduction by heterotrimeric G-proteins are covered first, followed by a section on effector molecules, secondary messengers, and protein kinases and phosphatases activated *via* heterotrimeric G-proteins. This section also includes some general features of protein kinases and phosphatases. The third section deals with transmembrane receptor kinases and signaling via monomeric G-proteins. This section includes the MAPK cascade. The fourth section deals with the regulation of intracellular levels of calcium and how calcium may play a role in signaling.

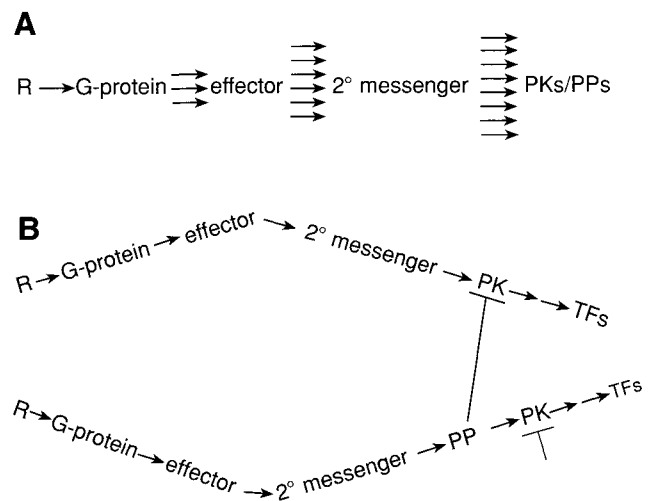


FIGURE 25-2 Multistep signaling allows amplification of signal (A) and cross talk among different signals (B).

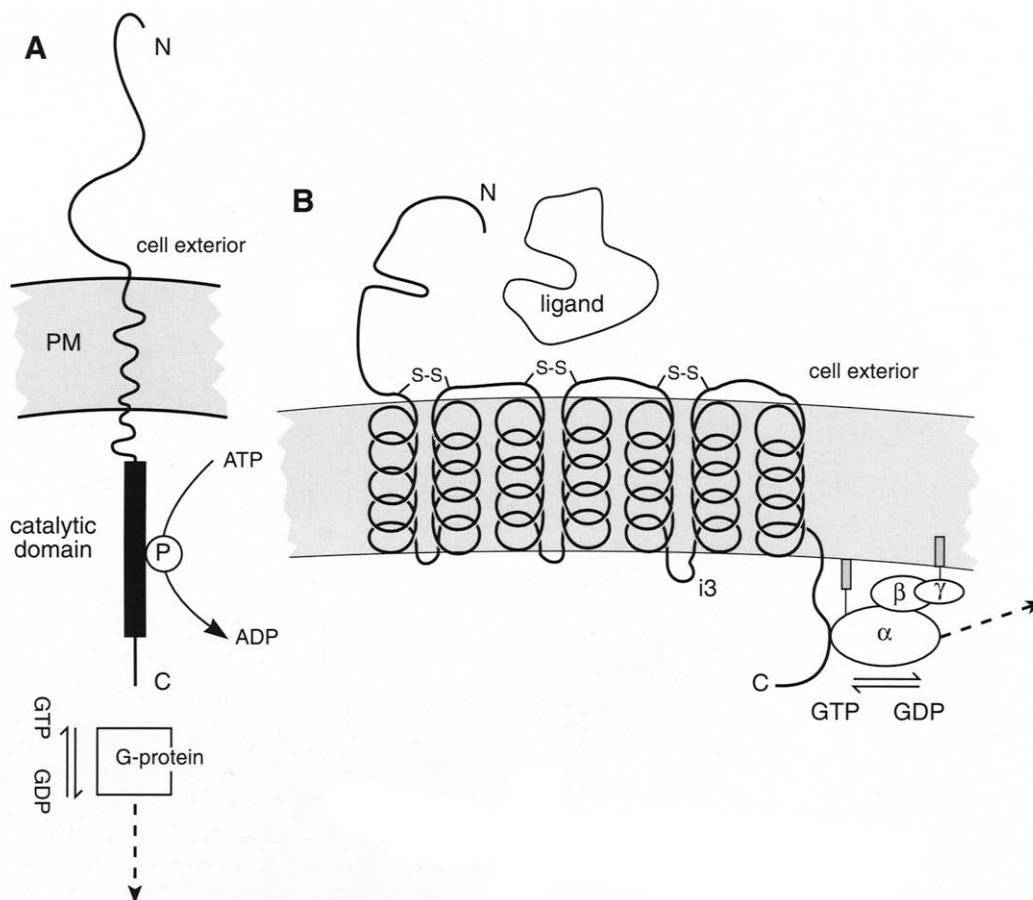


FIGURE 25-3 Two types of plasma membrane-based receptors. (A) A transmembrane receptor kinase carries the receptor function and kinase activity on the same polypeptide. (B) A seven-pass transmembrane receptor interacts with the GTP-binding subunit of a heterotrimeric G-protein. The extracellular N terminus of the seven-pass receptor is often glycosylated and, together with the transmembrane domains, which are stabilized by extracellular disulfide links, forms a ligand-binding pocket. The intracellular C-terminal and the inner loop 3 (i3) from the N-terminal are believed to activate the α subunit of a heterotrimeric G-protein.

SECTION I. G-PROTEIN-COUPLED RECEPTORS (GPCRS) AND HETEROTRIMERIC G-PROTEINS

1. GPCRS ARE A SUPERFAMILY OF INTEGRAL MEMBRANE PROTEINS

GPCRs are found in a wide range of organisms, including vertebrates, arthropods, insects, yeast, and slime mold. More than 700 members of this superfamily are known and specifically recognize a vast array of different types of ligands, visual pigments, olfactants, neurotransmitters, peptide hormones, insect pheromones, fungal mating factors, and molecules involved in chemotaxis. Despite this vast variety, these recep-

tors, based on sequence data from cDNA clones, show a highly conserved structure. They are characterized by seven hydrophobic regions, putative transmembrane domains, each about 20–28 amino acids in length and believed to form α helices, that are linked by alternate hydrophilic intra- and extracellular loops. The seven-transmembrane domains are stabilized by extracellular, but intramolecular, disulfide bonds and, with the extracellular domain, form a ligand-binding pocket (see Fig. 25-3B). These receptors are sometimes referred to as “serpentine” or 7-pass or 7TM receptors.

The intracellular hydrophilic regions, especially the inner loop 3 (loop i3) between the transmembrane, span V and VI (from the N-terminal), and the C terminus of the GPCR are believed to interact with the α subunit of the heterotrimeric G-proteins (see Section I,2). This assumption is based on several observations.

- i. Sequence diversity in loop i3 and the C terminus is greater than in other cytoplasmic domains of the GPCRs.
- ii. Chimeric and mutant receptors with deletions in loop i3 and/or C terminus indicate that these regions are important in GPCR and G-protein coupling.
- iii. Mastoparan, an amphipathic peptide with an α -helical structure, isolated from wasp venom, is structurally very similar to loop i3. It is able to activate certain classes of $G\alpha$ proteins in a receptor-like manner.

7TM receptors are highly diverse in their extracellular domains, which is expected in view of the diversity of ligands that they bind to or associate with. The intracellular domains, especially loop i3 and the C terminus, are also diverse because they interact with a variety of $G\alpha$ proteins (see later). Nonetheless, the membrane-spanning domains are conserved, and the sequence information can be used for a data-based search to isolate new members of this large superfamily.

2. HETEROTRIMERIC G-PROTEINS

Heterotrimeric G-proteins are so called because they are a complex of three separate proteins, $G\alpha$ ($M_r \sim 35\text{--}45\text{ kDa}$), $G\beta$ ($\sim 35\text{--}36\text{ kDa}$), and $G\gamma$ ($\sim 8\text{--}10\text{ kDa}$). The $G\alpha$ subunit acts as a GTPase, it binds and hydrolyzes GTP; the $G\beta$ and $G\gamma$ subunits, members of a family of dimeric proteins, have been recruited for functioning of the $G\alpha$ subunit.

2.1. Structures of $G\alpha$, $G\beta$, and $G\gamma$ Subunits

$G\alpha$ proteins, like other GTPases, are structurally highly diverse, except for five conserved domains, G1 through G5, which are involved in GTP binding and hydrolysis (Fig. 25-4). The variable parts include the C terminus, which interacts with the 7TM receptor and with the downstream effectors, and the N terminus, which interacts with the $G\beta\gamma$ dimer. $G\alpha$ subunits are prenylated and tethered to the plasma membrane by lipid chains near their N termini. Several classes, and many subclasses, of $G\alpha$ proteins are recognized based on their affinities for GDP-GTP, rates of GTP hydrolysis, and susceptibility to certain toxins (see later). They also differ in their tissue distribution, association with different 7TM receptors, and activate or inhibit specific effectors.

$G\beta$ subunits belong to the WD40 family of proteins. Members of this family have a variable N-terminal

region followed by multiple copies of a repeat motif, 4 to 16 copies of which can be present in a single protein. The motif consists of amino acid sequences of more or less constant length (~ 44 to 60 residues), which typically end with a tyrosine-aspartic acid (WD) pair at the C terminus of the sequence; hence, the "WD" repeat (Fig. 25-5). $G\beta$ proteins typically have seven repeats, which form antiparallel β sheets making up the blades of a three-dimensional propeller-like structure. The WD repeat occurs in many proteins of diverse functions, which are believed to be involved in protein-protein interactions.

$G\gamma$ subunits are more diverse in structure. Their internal sequences determine their association with different $G\beta$ units. Each $G\gamma$ subunit is prenylated and carries a lipid chain at the C terminus, which anchors the $G\beta\gamma$ dimer and the inactive trimer to the plasma membrane.

2.2. Signal Transduction

The biochemistry of signal transduction is well characterized.

- i. In the inactive state, the $G\alpha$ subunit is bound to GDP and to the $G\beta\gamma$ dimer. Native trimers are tethered to the cell membrane *via* short lipid chains at the C terminus of the γ subunit and the N terminus of the α subunit (Fig. 25-6A).

- ii. The 7TM receptor, on binding to its ligand, undergoes a conformational change, which activates the α subunit to shed GDP and exchange it for GTP. The GTP-bound form of the heterotrimer is unstable and dissociates to form GTP- α and $\beta\gamma$ complexes (Fig. 25-6B).

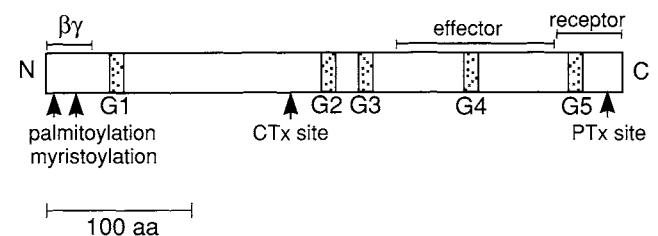


FIGURE 25-4 Schematic structure of a $G\alpha$ protein. N and C termini of the polypeptide are indicated along with an approximate scale in amino acids. The five conserved regions (G1 through G5) loop together to form the guanine nucleotide-binding domain. $G\alpha$ subunits are lipid modified at their N termini through addition of a myristoyl and a palmitoyl moiety, which tethers them to the plasma membrane. Regions believed to bind to the $\beta\gamma$ dimer, downstream effectors, and C terminus of the cognate 7TM receptor are indicated. Sites that are targets for ADP-ribosylation by cholera (CTx) and pertussis (PTx) toxins are also indicated. They are present in some, but not all, $G\alpha$ subunits. Adapted with permission from Millner and Causier (1996) and Hooley (1999).

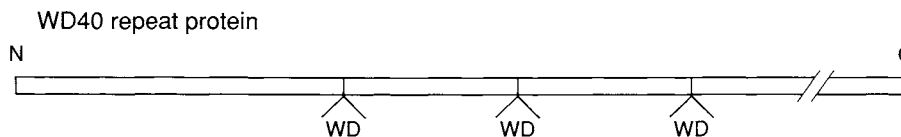


FIGURE 25-5 Schematic illustration of a protein with WD repeats. Only three repeats are shown. Adapted with permission from Smith *et al.* (1999).

iii. Acting either coordinately or independently, these two species bind and modulate the activities of downstream effector molecules (Fig. 25-6B). G-proteins are released from effectors upon the hydrolysis of GTP that results from the slow GTPase activity of the α subunit.

iv. The inactivated α subunit, bound to GDP, reassociates with the $\beta\gamma$ dimer, reforming the trimer, which can then reassociate with its receptor and undergo a new cycle of signal transduction (Fig. 25-6C).

v. The rate of hydrolysis of GTP, determined by the type of $G\alpha$ protein involved, regulates the lifetime of the interaction between the GTP- α or $\beta\gamma$ subunits and the effector molecules.

2.3. Combinations of Different $G\alpha$, $G\beta$, and $G\gamma$ Proteins Provide a Diversity of Heterotrimeric G-Proteins

Animals have a large number of heterotrimeric G proteins, which result from different combinations of isoforms of $G\alpha$, $G\beta$, and $G\gamma$ subunits. For instance, mammals have 20 $G\alpha$, 5 $G\beta$, and 7 $G\gamma$ subunits, which combine in various ways to interact specifically with hundreds of different receptors and more than a dozen effectors.

2.4. $G\alpha$ Proteins Are Slow GTPases

The transduction cycle is driven by a series of ligand-activated (receptor) and GTP-driven ($G\alpha$ protein) conformational changes that result in recognition of an effector and modulation of its activity. $G\alpha$ proteins act as catalytically slow GTPases, taking seconds for hydrolysis. Their “inefficiency” is programmed and designed to achieve optimal activation of a specific effector. For example, the $G\alpha$ subunit specific for adenylyl cyclase (see Section II.1.2) may activate a series of adenylyl cyclases in succession before the GTPase activity terminates the association. Thus, the

half-life of the GTP- α complex is not only geared to a specific effector, it is also timed to provide optimal signal amplification.

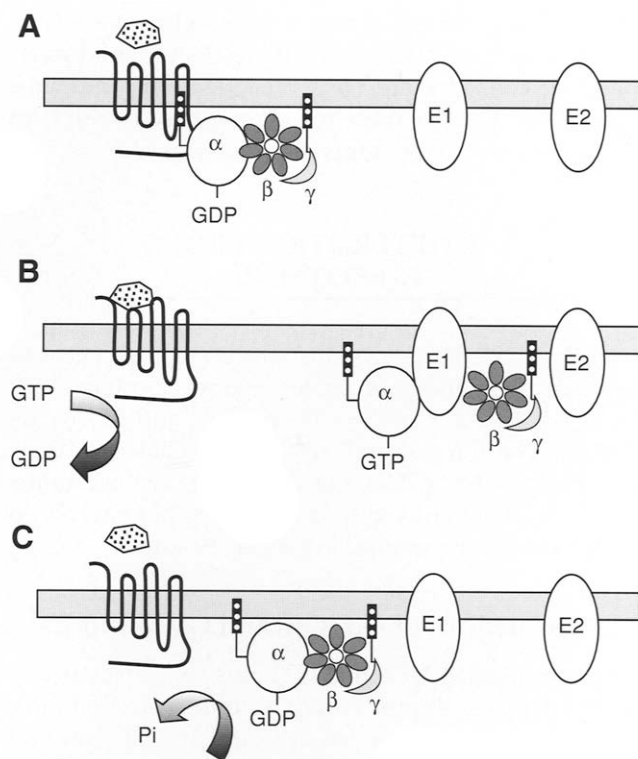


FIGURE 25-6 Stages in the G-protein catalytic cycle. (A) In the inactive state, the $G\alpha$ subunit is bound to GDP and to the $G\beta\gamma$ dimer. (B) On binding of the ligand, the activated receptor interacts with the $G\alpha$ protein, stimulating an exchange of GTP for GDP. The activated G-protein trimer dissociates into GTP- α and $\beta\gamma$ complexes. Each complex can interact with downstream effector molecules (E1 and E2). (C) Inherent GTPase activity of the $G\alpha$ subunit hydrolyzes the bound GTP, the inactivated $G\alpha$ binds to the $\beta\gamma$ dimer, and the reestablished heterotrimeric form is ready to enter another round of the cycle. Modified with permission from Hooley (1999).

BOX 25-1 DEMONSTRATION THAT HETEROTRIMERIC G-PROTEINS ARE PRESENT IN A CELL/TISSUE AND INVOLVED IN SIGNALING

THE PRESENCE OF G-PROTEINS in cells/tissues is demonstrated by a variety of methods.

- i. Assays for GTPase activity and specific binding of radiolabeled GTP to cell fractions are usually the first indications that such proteins might be present.
- ii. Additional evidence is provided by immunoblotting using antisera against known epitopes of G-proteins.
- iii. Evidence that G-proteins are functional in signaling is obtained by use of inactive homologues of GTP/GDP and certain bacterial toxins. GTP γ -S or GDP β -S are inactive homologues of GTP or GDP, respectively, where the terminal phosphate has been replaced by sulfate. GTP γ -S can bind to the G α subunit, but cannot be hydrolyzed; hence, it keeps the signal firing continuously. The GDP β -S binds to G α , but does not allow it to be activated by the 7TM receptor. Two toxins secreted by bacteria, pertussis toxin and cholera toxin, inactivate certain classes of G α subunits. They do so by adding ADP-ribose to specific amino acid residues in G α subunits (see Fig. 25-4), a process known as ADP-ribosylation. ADP-ribosylation by cholera toxin freezes the G α subunit in the active, GTP-bound state, which results in a continuous firing of the signal. In contrast, pertussis toxin freezes the G α subunit in the GDP-bound or an inactivated state. These toxins, alone or in combination with GTP γ -S or GDP β -S, can be used with plasma membrane or microsomal preparations or injected in intact cells or tissues to demonstrate whether a particular response is being affected. If it is, that is a positive indication that signaling by heterotrimeric G-proteins is involved.
- iv. Mastoparan, an amphipathic peptide, has been used to provide evidence of G-protein activation by 7TM receptors. As indicated earlier, it is thought to mimic the 7TM receptor action and activate G α subunits of the heterotrimeric G-proteins. If a well-characterized response can be obtained by a supply of Mastoparan, in the absence of the signal (ligand), that is considered an indication that 7TM receptors are present and signaling is *via* heterotrimeric G-proteins. However, Mastoparan is not specific to G α ; also, it is known to cause changes in membrane permeability, and the response obtained can be due to ion leakage.

3. 7TM RECEPTORS AND HETEROTRIMERIC G-PROTEINS IN PLANTS

3.1. Genes for GPCR-like Proteins Have Been cloned in Plants

Since several regions of genes encoding 7TM receptors are highly conserved, a search for predicted proteins with these conserved motifs in databases for expressed sequence tags (ESTs) can reveal candidate sequences. One sequence from *Arabidopsis* so isolated and named *GCR1* (for G-protein coupled receptor1) encodes a very large protein, more than 5000 amino

acids. Several other sequences in *Arabidopsis*, oilseed rape, pine, wheat, and hybrid aspen, have been identified also, but their functions are unknown.

3.2. Occurrence of Heterotrimeric G-Proteins in Plants

The presence of heterotrimeric G-proteins, particularly G α proteins, has been inferred from a range of pharmacological, physiological, and immunological studies. For instance, plasma membrane and microsomal fractions from several plants show high-affinity specific binding of radiolabeled GTP and GTP γ -S and yield polypeptides that can be ADP-ribosylated by cholera and pertussis toxins. Antisera directed against

animal $G\alpha$ or $G\beta$ subunits have been used to detect the presence of similar proteins in plant extracts. Activity of the inward-directed K^+ channel in guard cells is modulated by the inactive sulfated analogs of GTP and GDP, by the bacterial toxins, and by Mastoporan, which activates GDP-GTP exchange by the $G\alpha$ subunit in a receptor-like manner. Although the exact manner in which the putative $G\alpha$ modulates the K^+ channel is not understood, its modulation by these agonists and antagonists is suggestive of G-protein involvement. Similarly, heterotrimeric G-proteins have been implicated in IAA signaling in rice coleoptiles, in gibberellin induction of α -amylase genes in aleurone tissue, in plant responses to blue and red light, and in responses to pathogens and fungal elicitors.

3.3. DNA Sequences Encoding $G\alpha$, $G\beta$, and $G\gamma$ Proteins Have Been Cloned

Compelling evidence comes from cloning of the genes encoding the individual subunits. The first gene encoding a $G\alpha$ protein [*GPA1* (for G-protein alpha1)] was cloned in *Arabidopsis* in 1990. Since then, homologues have been cloned from tomato, maize, rice, soybean, lotus, and several other plants. All plant $G\alpha$ proteins whose cDNAs have been cloned to date show the five conserved domains, typical of all GTPases (see Fig. 25-4). They also show a very high sequence identity among themselves and evolutionarily seem to fall in a group distinct from animal $G\alpha$ proteins. mRNA and reporter gene expression and immunolocalization of the expressed protein suggest that the genes are expressed throughout development and in all major organs, but particularly so in younger tissues. Intracellularly, the protein seems to be located at the plasma membrane and in the endoplasmic reticulum.

cDNAs and genes encoding $G\beta$ subunits have been cloned from *Arabidopsis*, oat, rice, maize, and tobacco, and show the conserved WD40 repeat motif. The cDNA for a putative $G\gamma$ subunit has also been cloned from *Arabidopsis*.

Thus, plants do have genes encoding the subunits of heterotrimeric G proteins. However, in contrast to mammalian genomes, which usually have multiple genes for each subunit, plant genomes show only one or two genes encoding each subunit. Such parsimony suggests that heterotrimeric G proteins in plants are involved in a limited number of signaling pathways.

Although the pharmacological evidence from the use of Mastoparan, inhibitors, and sulfated analogs of GTP and GDP may be subject to differing interpretations, genetic evidence for a role for a plant $G\alpha$ protein has been provided also. Mutations in a gene

encoding a $G\alpha$ protein in rice yield a dwarf phenotype with dark green foliage and small rounded seeds, in contrast to the wild phenotype which is tall and bears larger, elongated seeds. The mutant phenotype is also mimicked by an antisense suppression of the $G\alpha$ gene. This suggests that the same $G\alpha$ protein may have pleiotropic roles in plant development.

4. SECTION SUMMARY

Perception of extracellular signals by 7TM receptors and transduction of the signal *via* heterotrimeric G-proteins is an established theme conserved in yeast and animal systems. That such signaling may also occur in plants is indicated by the presence of sequences that encode putative 7TM proteins, and $G\alpha$, $G\beta$, and $G\gamma$ proteins. The evidence from pharmacological studies that heterotrimeric G-proteins may be involved in signaling for stomatal behavior, hormonal responses, and light- and pathogenesis-related processes has been buttressed by genetic and molecular evidence for a role for $G\alpha$ protein in rice development and morphogenesis. These are important achievements of the last decade in plant signaling, but major gaps remain. For instance, it has not yet been shown that the $G\alpha$, $G\beta$, and $G\gamma$ components actually form a heterotrimeric complex and/or dissociate into $G\alpha$ and $G\gamma$ dimer on GTP hydrolysis, that is, they function in signaling as in animal cells. Also, whether the 7TM proteins serve as receptors is an open question. Future studies will undoubtedly be directed to solving these problems.

SECTION II. OTHER ELEMENTS OF THE G-PROTEIN SIGNALING PATHWAY

1. EFFECTORS

Heterotrimeric G-proteins convey the signal to secondary messengers via effectors, which are either ion channels or enzymes.

1.1. Ion Channels

Ion channels, as explained in Chapter 13 (see Box 13-1) are proteinaceous pores in the plasma membrane or vacuolar membrane, or membrane of an organelle (e.g., chloroplast, mitochondria, or endoplasmic reticulum). They span the membrane and occur in "open" or "close" conformational states. They may

be specific to an ion, or nonspecific, and may be stretch, voltage, or receptor activated. As mentioned previously, pharmacological studies using bacterial toxins and inactive GTP/GDP homologues have provided evidence that heterotrimeric G-proteins are involved in regulation of the inward-directed K^+ channel in guard cells, although the exact signaling pathways are unclear (see Section I,3.2). Similar data are beginning to accumulate with regard to Ca^{2+} channel activity in plants.

1.2. Effector Enzymes and Secondary Messengers

Effector enzymes are responsible for regulating the intracellular concentrations of secondary messengers, such as cyclic AMP (cAMP), cyclic GMP (cGMP), diacylglycerol (DAG), inositol-1,4,5-triphosphate (IP_3), and calcium (Ca^{2+}), which, in turn, regulate the activities of many secondary messenger-dependent protein kinases or phosphatases.

Nucleotide cyclases and phosphodiesterases regulate the intracellular levels of cAMP and cGMP. Adenylate cyclase produces 3'5'-cyclic AMP from ATP, whereas adenylate phosphodiesterase degrades it to 5' AMP (Fig. 25-7). Similar guanylate enzymes regulate the levels of cyclic GMP. These enzymes and the second messengers they regulate play important and widespread roles in animal cells. One of the examples of signal amplification is provided by adenylate cyclases. A single receptor-hormone complex in animal cells causes the conversion of several $G\alpha GDP$ $G\beta\gamma$ complexes to the active $G\alpha GTP$ form, which in turn activates several adenylate cyclase molecules. Each of these enzymes catalyzes the synthesis of many cAMP molecules during the time that $G\alpha GTP$ is bound to it. Despite their importance in animal cells, cAMP and cGMP and the protein kinases they regulate seem to play only limited roles in plant cells (see Section II,2.1).

The intracellular levels of DAG and calcium are regulated by the coupling of G-protein signaling and the phosphoinositide pathway. Phosphatidylinositol (PI) is an abundant metabolite in most plant cells (for synthesis of some of the phospholipids in this section, see Box 12-2 in Chapter 12). It is phosphorylated in succession to give phosphatidylinositol phosphate (PIP) and phosphatidylinositol-4,5-bisphosphate (PIP_2). Phospholipase C is an effector enzyme, which occurs in multiple isoforms, both membrane bound and soluble. The β form, here called PLC, is the isoform activated by $G\alpha$ signaling. It occurs bound to the inner leaflet of the plasma membrane and, on activation, cleaves the phosphodiester bond between PIP_2 and DAG to give inositol-1,4,5-triphosphate. DAG is hydro-

phobic and stays bound to the inner leaflet of the plasma membrane, but IP_3 is water soluble and freely mobile. On release from PIP_2 , it diffuses to its receptor sites on vacuolar and ER membranes. The IP_3 receptor is a tetrameric transmembrane protein, which forms a Ca^{2+} -specific channel—the channel opens on binding of the ligand IP_3 . The NH_2 -terminal in the cytoplasm carries the IP_3 -binding site, while the transmembrane carboxy terminus forms the channel through which stored Ca^{2+} moves out into the cytoplasm (Fig. 25-8). It is not known whether all four sites need to be occupied by Ca^{2+} for the channel to open. PLC thus regulates the intracellular levels of free Ca^{2+} and DAG.

In recent years, phosphatidic acid (PA) and diacylglycerol diphosphate (DGPP) have also been implicated in plant signaling. PA is thought to be produced by the activity of another effector enzyme,

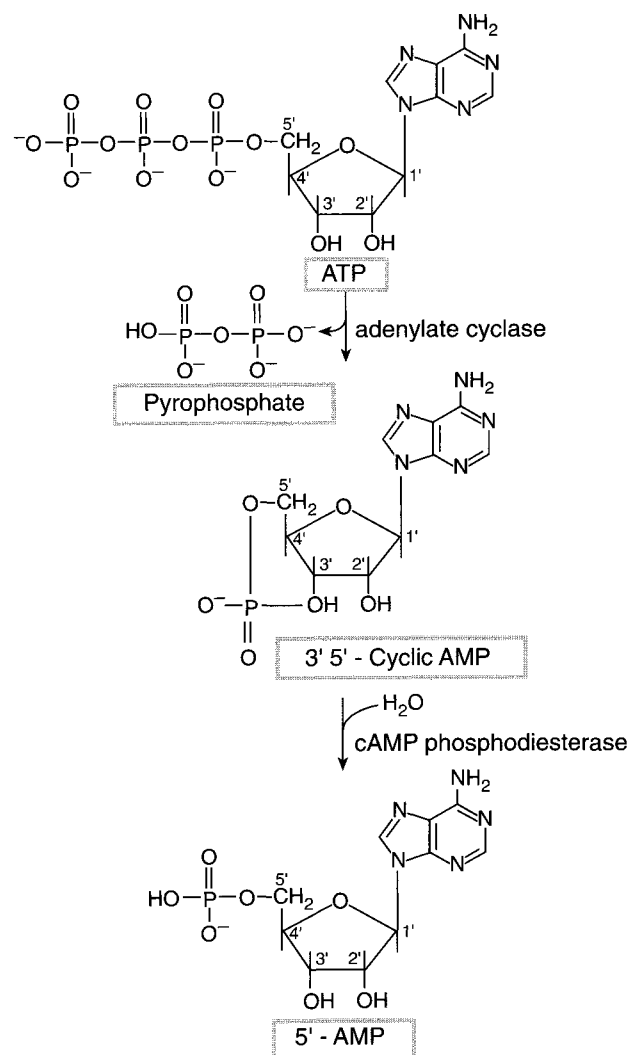


FIGURE 25-7 Synthesis and degradation of 3'5'-cyclic AMP.

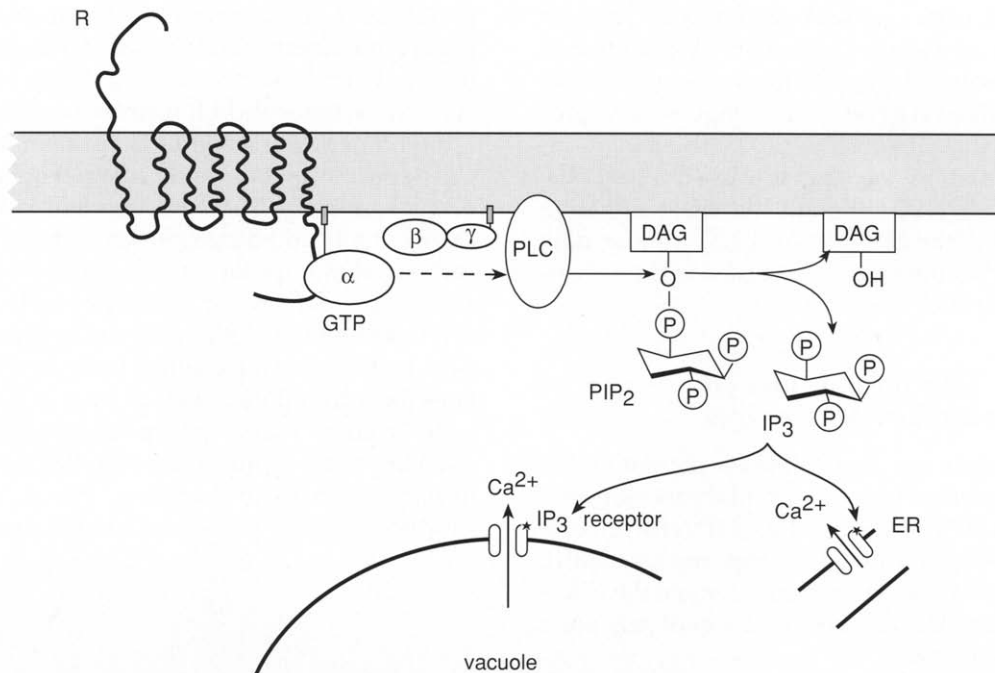


FIGURE 25-8 Phosphoinositide pathway and its coupling to heterotrimeric G-protein. The GTP- $G\alpha$ complex activates phospholipase C, which in turn breaks the phosphodiester bond between phosphatidylinositol-4,5-bisphosphate and diacylglycerol to give rise to inositol-1,4,5-trisphosphate (IP_3). IP_3 is freely mobile and migrates to its receptors, on ER and vacuolar membranes, which act as ligand-gated Ca^{2+} channels, leading to an increase in the level of free cytosolic Ca^{2+} . One of the IP_3 -binding sites on the Ca^{2+} channel polypeptides is shown by an asterisk. Modified with permission from Drøbak (1992).

phospholipase D (PLD). It can also be produced from DAG. PA is further phosphorylated to DGPP. Like PLC, PLD is activated in response to a variety of signals, especially after pathogen attack, wounding, or water stress. A PLD gene has been shown to be one of the first ones expressed in the resurrection plant *Craterostigma plantagineum* subjected to drought. Experiments using radiolabeled ^{32}P i indicate that the levels of these two phospholipids are elevated rapidly following water stress. The precise connection among G-protein signaling, induction of PLD, and the targets of PA and DGPP signaling are unclear, however.

Calcium is an important second messenger in plants with many roles in physiology and morphogenesis (see Section IV). It is also known to modulate the activities of calcium-dependent and/or calcium-calmodulin-dependent enzymes, covered later in this section, and for modulation of plant 14.3.3 proteins (see Box 25-3). In contrast, diacylglycerol, like cAMP and cGMP, seems to play only a limited role in plant signaling.

Before discussing secondary messenger-dependent protein kinases, it is important to emphasize the role of protein phosphorylation and dephosphorylation in plant growth and development and some important features of protein kinases and phosphatases.

2. PHOSPHORYLATION/DEPHOSPHORYLATION OF PROTEINS IS A MAJOR STRATEGY ADOPTED BY ORGANISMS

Phosphorylation/dephosphorylation of proteins is a major strategy adopted by organisms for their responses to environmental, developmental, and metabolic signals. Protein kinases transfer the terminal (γ) phosphate group from ATP (less commonly GTP) to one or more specific amino acids in the target protein. The phosphorylated amino acids are either serine/threonine or tyrosine, and the kinases are classified as serine/threonine kinases or tyrosine kinases, respectively. Some kinases are a mixed bag. They show dual specificity and are able to phosphorylate both serine/threonine residues and tyrosine residues on the same polypeptide. The protein phosphatases dephosphorylate the same amino acid residues. Protein kinases and protein phosphatases thus act as the "yin" and "yang" of signaling and change the phosphorylation status of structural proteins, regulatory proteins, and enzymes in a highly specific manner. They activate/deactivate cyclin-dependent kinases

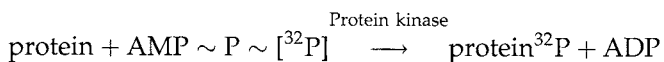
(CDKs) involved in cell division, transcription factors involved in gene expression, and enzymes involved in metabolic pathways. Thus, they mediate signal transduction pathways leading to growth, gene expression, and metabolic control.

Just as tow trucks tow trucks, and also tow tow trucks, protein kinases phosphorylate other kinases and are dephosphorylated by protein phosphatases, and *vice versa*. In some cases, there are cascades of phosphorylations/dephosphorylations, where a series of kinases phosphorylate other kinases in the series by donating their phosphate group.

A large number of protein kinases and phosphatases occur in eukaryotic cells, which provide a vast repertoire for the modulation of specific signaling pathways by specific kinases and/or phosphatases. The number of responses that can be regulated in this manner is immense. At the same time, phosphorylation/dephosphorylation steps provide ample opportunities for signal amplification or attenuation, for transmission of signal from cell surface receptors to cell interior and nucleus, and for cross talk between different signaling pathways.

Protein kinases show variable structures, but an ~ 300 amino acid catalytic domain, with alternating conserved (I–XI) and not so conserved subdomains, is common to all protein kinases (Fig. 25-9). The catalytic domain provides specificity for substrates and whether serine/threonine or tyrosine residues are phosphorylated, whereas the regions outside the catalytic domain provide for regulation of the catalytic activity. In some cases, the regulatory subunit(s) is a separate polypeptide, which together with the catalytic subunit, forms the functional holoenzyme; in others the catalytic and regulatory domains occur on the same polypeptide.

The presence of protein kinases is usually demonstrated by incubating cell/tissue extracts with ATP radiolabeled with [^{32}P] in the γ position.



Various inhibitors of protein kinases are known (e.g., staurosporine, K252a, chelerythrine). Inhibition of a particular response by an inhibitor provides indirect evidence that protein kinases are involved in the response pathway. Some inhibitors are more specific to a particular class of protein kinases than others. For example, some triterpenoids or isoflavones specifically inhibit the cAMP-activated protein kinase A, but not calcium-dependent protein kinases.

Since the first cloning of a protein kinase gene from common bean in 1989, the number of protein kinases known from plants has increased dramatically. They have been grouped into families based on structural characteristics, substrate specificity, regulatory ligand, and cellular function. One such grouping is shown in Fig. 25-10.

We now return to the protein kinases that are known to be regulated by secondary messengers, although it must be realized that such distinctions are somewhat arbitrary because ultimately all phosphorylations or dephosphorylations are a consequence of some signal.

2.1. The AGC Group of Protein Kinases

The “AGC” group of protein kinases is activated by secondary messengers. Cyclic AMP and cyclic GMP activate protein kinase A and protein kinase G, respectively, whereas phosphatidylserine and Ca^{2+} , in association with diacylglycerol, activate protein kinase C. The AGC group of protein kinases and their regulators are common in animal systems and simple eukaryotes (e.g., yeast, slime molds), but in plants, despite intensive search, they have been found only sporadically and their roles in signaling are not firmly established.

There are a few references to kinases that could be activated by phosphatidylserine and Ca^{2+} or cyclic AMP, which suggests the presence of protein kinase C- or A-like kinases in plant extracts. Some kinase genes have been cloned from a few plants (bean, pea,

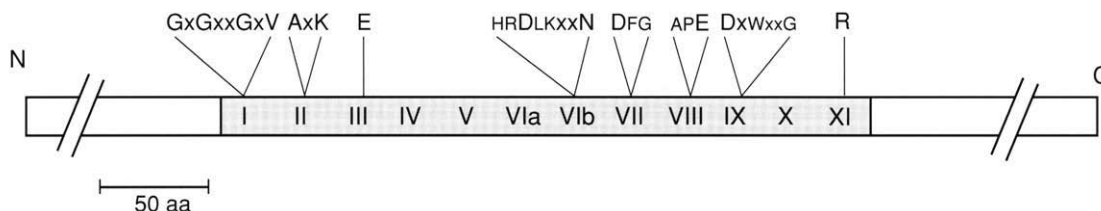


FIGURE 25-9 The catalytic domain of protein kinases shows 12 conserved subdomains (shown by Roman numerals I through XI), which alternate with not so conserved subdomains. From Stone and Walker (1995).

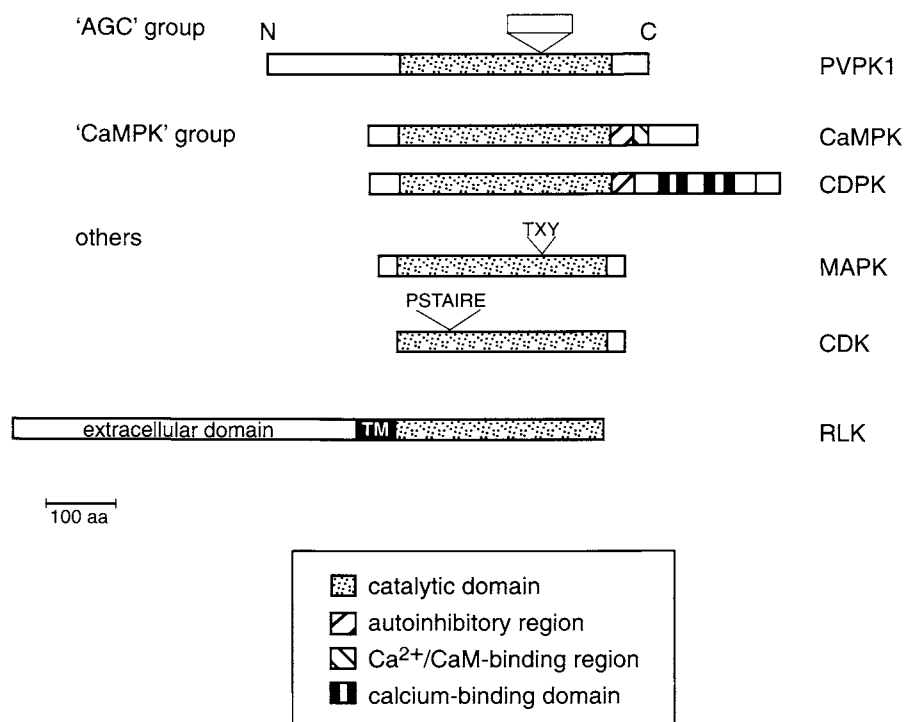


FIGURE 25-10 Schematic drawings of some major families of plant protein kinases showing their relevant features. Kinase catalytic domains are indicated by a shaded region. Plant protein kinases of the AGC group identified to date carry an 80–85 amino acid insert in the catalytic domain. Calcium–calmodulin-dependent protein kinases (CaMPKs) have an N-terminal catalytic domain, which is separated from the CaM-binding domain by an autoinhibitory region, which keeps the catalytic domain inaccessible to substrates until Ca²⁺/CaM is available. On binding to Ca²⁺/CaM, autoinhibition is released. Calcium-dependent protein kinases (CDPKs) have a similar structure except that there is no CaM-binding region. Instead, the C-terminal itself serves to bind Ca²⁺ in a CaM-like manner. Other protein kinase include mitogen-activated protein kinases (MAPKs), cyclin-dependent kinases (CDKs), and receptor-like kinases (RLKs). Modified from Stone and Walker (1995).

potato) that encode catalytic domain sequences similar to those in vertebrate protein kinases of the AGC group. The plant kinases, however, have an 80 or 85 amino acid insert in the catalytic domain (see Fig. 25-10). The functions of these kinases are not known, although some genes from pea (*PsPk1–PsPk5*) were shown to be down- or up-regulated in light and to be implicated in phytochrome signaling. Some DNA-binding nuclear proteins from tobacco (e.g., TGA1a and TGA1b) show sequence similarity to some cAMP-dependent kinases from mammalian systems. Pharmacological studies, using whole cell patch clamp technique, suggest that cyclic AMP and protein kinase A regulate the outward-directed K⁺ current in *Vicia faba* mesophyll protoplasts, and, as mentioned earlier, use of bacterial toxins and inactivated GTP–GDP homologues has indicated involvement of heterotrimeric G-proteins in regulation of the inward-directed K⁺ channel in guard cells of the same plant. Cyclic GMP has also been detected in plants, and some phytochrome responses, such as greening of

etiolated seedlings and anthocyanin accumulation, are thought to be induced *via* heterotrimeric G-proteins and cyclic GMP, but whether protein kinase G is involved is not known.

In conclusion, there is still only patchy evidence that cyclic AMP or cyclic GMP and their-dependent protein kinases occur in appreciable quantities or are major signaling molecules in plants. Recently, cAMP was shown to be involved in pollen tube growth and a cDNA believed to be encoding an adenylate cyclase was isolated from pollen grains of *Agapanthus umbellatus*. If confirmed, this would be the first cloning of a plant adenylate cyclase gene and may open more fruitful research into the biological roles of these secondary messengers.

In contrast to the AGC group, protein kinases that are regulated by calcium either directly or indirectly *via* calmodulin are abundant in plants. The Ca²⁺/calmodulin activation of target proteins is considered first.

2.2. Ca^{2+} /Calmodulin Activation

Calmodulin (CaM) belongs to a superfamily of Ca^{2+} -binding proteins (see Box 25-2). CaM has no enzyme activity itself, but on binding to Ca^{2+} under-

goes a conformational change and binds to and activates target proteins, usually protein kinases or protein phosphatases, in a calcium-dependent manner.

BOX 25-2 CALCIUM-BINDING PROTEINS

MOST PROTEINS THAT FUNCTION as intracellular transducers of Ca^{2+} signals contain a common structural motif, the "EF hand." These motifs typically occur in closely linked pairs, which form the basis for cooperativity in Ca^{2+} binding. A typical EF hand consists of two α helices, more or less at right angles to each other, connected by a loop that holds the Ca^{2+} ion; hence, the designation helix-loop-helix. The designation EF hand stems from the crystal structure of parvalbumin, a calcium-binding protein from animals. Parvalbumin contains three Ca^{2+} -binding domains, designated AB, CD, and EF, from the N terminus. The letters refer to α helices. The last domain, the EF domain, is the one for which the proteins were named.

The superfamily of EF hand proteins is divided into several classes based on differences in the number and organization of EF hand pairs, amino acid sequences within or outside the motifs, and affinity to Ca^{2+} and/or selectivity and affinity to target proteins.

Calmodulin is an acidic EF hand protein present in all eukaryotic organisms (Fig. 25-11). The prototype CaM is a small polypeptide (~ 148 amino acid residues, $M_r \sim 16.7$ kDa) with two globular domains connected with a long flexible helix. Each globular domain contains a pair of intimately linked EF hands. Ca^{2+} is held in the site in coordination with oxygen atoms and is bound reversibly, with a K_d (dissociation constant) of 10^{-5} to 10^{-6} M. Apparently, all four sites need to be occupied by calcium ions. There are also some indications that the presence of target protein facilitates Ca^{2+} binding of CaM.

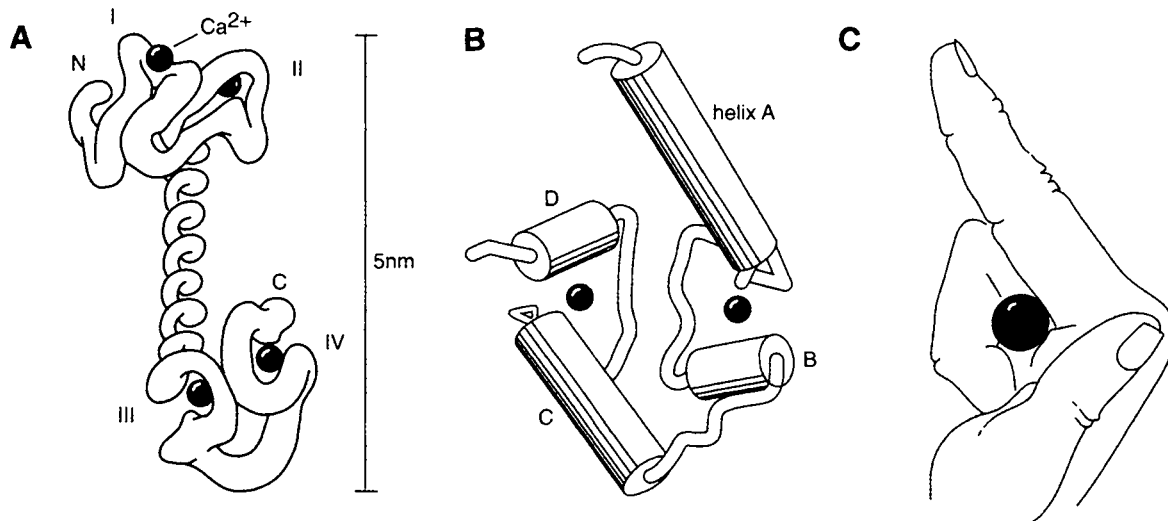


FIGURE 25-11 Schematic drawings showing calmodulin, the helix-loop-helix structure, and the EF hand. (A) A three-dimensional model of CaM showing two calcium-binding domains at either end (numbered I–IV) connected by an eight-turn α helix. From Alberts *et al.* (1994). (B) Helices are shown as rods (A–D) connected by loops. One pair of helices with the connecting loop forms the Ca^{2+} -binding site. Only two pairs, AB and CD, are shown. (C) The Ca^{2+} -binding site can be represented by the index finger and thumb (of the right hand), representing E and F α helices, while the middle finger forms the loop; hence, the designation EF hand. From Moncrief *et al.* (1990) and Strynadka and James (1989).

2.2.1. *CaM Genes in Plants and Their Expression*

Many *CaM* genes have been cloned from higher plants. Sequence comparisons indicate that they belong to large gene families with 6–12 members each. The encoded proteins (~ 16.7 – 16.8 kDa) show that the Ca^{2+} -binding domains are highly conserved, especially domain III. Domain IV is the most variable and seems to be involved in binding to the target proteins. Data from immunolocalization of the protein, as well as expressed mRNA and reporter gene, indicate that the protein is present in nearly all organs, more so in young meristematic tissues, such as root and shoot tips and intercalary meristems, than in mature tissues. The protein occurs in the cytosol in concentrations that have been estimated at ~ 5 to $20 \mu\text{M}$, but can be more or less.

The genes are expressed in response to various factors, such as light, pressure (touch or wind), wounding, and hormonal treatments (e.g., auxin, GA, or ABA treatments). Many of the same treatments have also been reported to elevate the levels of free cytosolic calcium in plant cells. For example, in *Arabidopsis*, several *TOUCH (TCH)* genes are induced by touch, pressure, rain and other mechanical stimuli. One of them, *TCH1*, encodes a *CaM* protein, whereas two others, *TCH2* and *TCH3*, encode *CaM*-related proteins. Pressure and wind cause changes in concentration of intracellular cytosolic calcium, and it is possible that a rise in cytosolic calcium goes hand in hand with an induction of *CaM* genes, but the nature of Ca^{2+} /*CaM*-modulated proteins in pressure-related responses is not known.

In animal systems, the Ca^{2+} /*CaM* complex regulates the activities of many protein kinases, designated *CaMPKs* (see Fig. 25-10). They include phosphorylase kinase, which phosphorylates other kinases, adenylate cyclase, plasma membrane-bound Ca^{2+} -ATPase, nicotinamide adenine dinucleotide (NAD) protein kinase, myosin light chain kinase, etc. It also activates phosphodiesterases and protein phosphatases such as calcineurin. In the absence of Ca^{2+} /*CaM*, the kinases are inactive. The presence of *CaM* is essential for many functions, and elimination of calmodulin gene in fungi has been shown to be lethal.

Although *CaM* genes are abundant in plants, functions of the Ca^{2+} /*CaM* complex in regulating kinases or phosphatases (or other proteins) in plants are still not clearly understood. There are many reports of Ca^{2+} /*CaM*-modulated kinase activities. Ca^{2+} /*CaM* complexes are reported to bind to transcription factors, to cytoskeletal proteins [e.g., microtubule-associated proteins (MAPs)] in connection with organ-

ization of mitotic spindle, and to actin microfilaments involved in cytoplasmic streaming. They are also implicated in phytochrome signaling, in regulation of K^{+} channel activity in guard cells, in regulation of Ca^{2+} -ATPases in both the endoplasmic reticulum and plasma membranes, and in hormonal responses. However, the details of interacting molecules, are unclear.

2.3. Calcium-Dependent Protein Kinases

In contrast to *CaMPKs*, which require a Ca^{2+} /*CaM* complex to activate them, protein kinases that combine the functions of Ca^{2+} binding and kinase activity in the same polypeptide, calcium-dependent protein kinases, are widespread in plants. They also occur in some protists, but are unknown from animals.

CDPKs have an N-terminal domain that acts as a serine-threonine protein kinase and a C-terminal that shows a *CaM*-like structure with four helix-loop-helix motifs that bind Ca^{2+} (see Fig. 25-10). The Ca^{2+} -binding domain is linked to the N-terminal by a junction domain, which acts as an autoinhibitor — the inhibitor binds to the kinase active site of CDPK in the absence of Ca^{2+} . On binding of Ca^{2+} , the inhibition is removed and the kinase domain is activated. Such autoinhibitory regulatory mechanisms are common to many second messenger-dependent protein kinases and phosphatases, including *CaMPKs*. Some *in vitro* assays indicate that the kinase activity of CDPKs may be stimulated 70 to 100-fold by free Ca^{2+} , but exogenous *CaM* has no effect.

CDPKs are widely distributed in both lower and higher plants and in various plant parts and cultures. They are reported in both soluble and membrane fractions and in association with chromatin in the nucleus. They have been shown immunocytochemically to colocalize with actin microfilaments in *Tradescantia* pollen tubes and internodal cells of *Chara*. In both types of cells, a Ca^{2+} -dependent actomyosin is implicated in the directional movement of organelles or vesicles.

Several CDPK genes have been cloned, at least 13 from *Arabidopsis*, also from soybean (where it was first isolated and sequenced), corn, carrot, peanut, etc. The M_r values calculated from several full-length cDNA clones predict proteins in the ~ 55 - to 60 -kDa range. Like *CaM* genes, they belong to multigene families, which show tissue and developmental specificity. For example, two CDPK proteins in *Arabidopsis* were constitutively expressed at low levels, as shown by immunolocalization, in almost all young parts—root and shoot

meristems, pericycle, and developing flowers—but were little or not at all expressed in older, or terminally differentiated tissues. The multiplicity of genes and different locations of CDPKs in plant tissues/parts and in cells (e.g., plasma membrane, chromatin, cytoplasm, microfilaments) suggest that this enzyme family is involved in multiple signal transduction pathways. However, despite their wide occurrence and obvious catalytic importance, very few natural substrates of CDPKs are known. Nodulin 26, a transmembrane protein assumed to play a role in metabolite transport across the symbiosome membrane in N_2 -fixing legume root nodules, and a plasma membrane based H^+ -ATPase have been shown to be phosphorylated by CDPKs. CDPKs phosphorylate histones in chromatin in *in vitro* assays and probably also *in vivo*. Several globular storage proteins in seeds are multimeric, composed of small and large chain polypeptides. CDPKs have been shown to phosphorylate both types of chains in napin-type storage proteins in *Brassica* and some other plants before their assembly into the holoprotein. Curiously, these chains act as antagonists of Ca^{2+} /CaM-modulated phosphorylation.

In summary, CDPKs seem to be involved in calcium-mediated phosphorylation reactions in young meristematic tissues and possibly with actin cytoskeleton rearrangements associated with tip growth and cytoplasmic streaming. They may also be involved in vesicular traffic in connection with transport of materials across cells.

2.4. Protein Phosphatases

Mammalian protein phosphatases (PPs) are classified into two types, PP1 and PP2, and type 2 is further divided into three subgroups, PP2A, 2B, and 2C, based on substrate specificities, sensitivity to inhibitors, subunit structure, and regulation by divalent cations

(Table 25-1). The catalytic subunits of PP1, PP2A, and PP2B are products of distinct genes, which associate with one or more regulatory subunits to form a holoenzyme. For example, in PP2A the catalytic subunit and the regulatory subunit A form a core dimer, which interacts with one of several variable B subunits (Fig. 25-12). Three families that encode these variable B subunits, B, B', and B'', are known with multiple isoforms in each, thus providing numerous different combinations for holoenzymes.

Calcineurin, a PP2B-type phosphatase, is specifically activated by Ca^{2+} /CaM; its activity is dependent on secondary messengers. PP2C is a monomer and has regulatory and catalytic domains on the same polypeptide.

Protein phosphatases are inhibited by several toxins. For example, okadaic acid (OA) and calyculin A, both produced by marine sponges, are potent inhibitors of PP2A. OA is a commonly used inhibitor. It has an IC_{50} of about 0.1–1.0 nM for PP2A, requires about a 100 times higher concentration to inhibit PP1, is marginally effective against PP2B, and has no effect on PP2C. Cyclosporin A specifically targets PP2B and is ineffective against PP2C.

2.4.1. Phosphatases in Plants

All four types of PPs have been identified, and genes encoding catalytic and/or regulatory subunits for PP1 and PP2A holoenzymes, or for PP2C, have been cloned from several plant species. PP1 activity has been recorded in *Brassica napus* seed extracts, in cytosol of pea leaves, in isolated nuclei, and in plasma membranes. *Arabidopsis* has a family of five genes encoding the catalytic subunit of PP1; at least one of them seems to be involved in cell cycle control, where it activates a cyclin-dependent kinase (CDK) by dephosphorylation.

TABLE 25-1 Subunit Composition, Cation Requirements, and Inhibitors of PPs^a

Phosphatase type	Subunit composition ^b	Cation required	Inhibition by	
			Okadaic acid	Cyclosporin
PP1	Di- or trimer, 2 or 3R/1C	—	++	?
PP2A	Trimer, 2R/1C	None	+++	?
PP2B	Dimer, 1R/1C	Ca^{2+}	+	++++
PP2C	Monomer with both R and C functions	Mg^{2+}	—	—

^aBased on data from Smith and Walker (1996).

^bR and C refer to regulatory and catalytic subunits.

PP2A has also been identified in a number of plant extracts and membranes. cDNAs and/or genes for the catalytic and regulatory subunits have been cloned in *Arabidopsis*, and gene-specific probes for regulatory subunits indicate that they are expressed in a tissue- and development-specific manner. PP2As are the principal enzymes for dephosphorylation and activation of many protein kinases in animal systems, including kinases in the MAPK cascade, CDKs, and CaMPKs. They probably serve similar functions in plants. In addition, they are thought to dephosphorylate and activate metabolic enzymes (e.g., sucrose phosphate synthase, nitrate reductase, phosphoenolpyruvate carboxylase, HMG-CoA reductase). A PP2A in *Arabidopsis* is also involved in normal root and hypocotyl elongation because a mutation in its A subunit (*rcn1* mutation) causes abnormal root curling and lack of apical hook formation in seedlings.

Inhibitors of PP2As have been used to show that phosphatases are involved in regulating K^+ flux in guard cells. Calcineurin, a PP2B-type phosphatase, which is specifically inhibited by cyclosporin A, was shown to block Ca^{2+} -induced inhibition of the inward-directed K^+ channel in guard cells.

Several genes encoding PP2C type proteins have been cloned from *Arabidopsis*. The best known are the ABI1 and ABI2 proteins, which are involved in various ABA-induced responses, including stomatal regulation, stress tolerance, seed germination, and dormancy, although exactly how these phosphatases regulate ABA signaling is not clear (but see Chapter 23). Another PP2C phosphatase from *Arabidopsis*, PP2C-At, was identified as a complementary rescue

for a yeast mutant that was deficient in cAMP phosphodiesterase. While the role of PP2C-At in *Arabidopsis* is unknown, it is yet another illustration of the possible involvement of cAMP-dependent phosphorylation cascade in plant signaling.

3. SUMMARY

Signal transduction from heterotrimeric G-proteins to protein kinases and phosphatases via the effectors and secondary messengers is still very much a black box in plants. The various components of this signaling are known from plants to varying extents, some are more abundant than others and some are unique to plants. The phosphoinositide pathway is operative in plants, IP_3 is produced, and Ca^{2+} is abundant in plants, but the other second messengers, cAMP, cGMP, diacylglycerol, and phosphatidyl serine, and the protein kinases, A, G, and C, that they activate seem to be present only sporadically and in modest amounts. Their identification has been mostly by immunological or biochemical means using antisera and inhibitors, which may not be conducive to detecting small amounts. Using a homology-based technique, a family of genes has been cloned that encode kinases with catalytic domains similar in sequence to those of the AGC group, and using gene-targeted mutagenesis, it may be possible to identify their functions. Calcium-binding protein, calmodulin, occurs ubiquitously in plant cells. Many genes encoding CaM have been cloned. They are expressed primarily in young meristematic tissues and are induced rapidly by many environmental as well as hormonal signals, but their substrate enzymes are still very much a mystery. Likewise, Ca^{2+} /CaM regulated protein kinases (CaMPKs) seem to play a limited role in plants. Instead, plants seem to have an abundance of a novel type of calcium-dependent protein kinases (CDPKs), which combine the functions of Ca^{2+} -binding and kinase activity in the same polypeptide. Many different CDPKs occur in plants; they are encoded by multigene families, and the members likely show tissue- and development-specific expression. Our knowledge of the natural substrates of CDPKs is still meager. Protein phosphatases have come to be recognized as important components of signaling in their own right. They occur in multiple forms, which differ structurally in substrate choice, cofactor requirements, and inhibitors. Plants possess all major types, and genes or cDNAs for many of them have been cloned, but the precise roles of these phosphatases in signaling are unclear because, for the most part, their substrates are unknown. Two phosphatases of type PP2C, ABI1 and ABI2, are especially noteworthy because they are involved in ABA

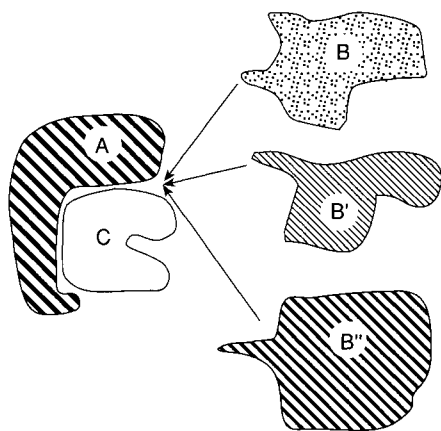


FIGURE 25-12 Protein phosphatase 2A (PP2A) holoenzyme. The catalytic subunit (C) is bound to regulatory subunit A. This heterodimer further complexes with any one of an array of B regulatory subunits, belonging to one of three families, B, B', or B''. From Millward *et al.* (1999) with permission from Elsevier Science.

signaling. Since PKs and PP_s operate upstream of gene expression, if a PK or PP is known to be involved in a specific signaling event, its constitutive expression in the target tissue can bring about the expected response in the absence of the signaling stimulus.

SECTION III. TRANSMEMBRANE RECEPTOR ENZYMES, SMALL G-PROTEINS, AND MAPK CASCADES

1. TRANSMEMBRANE RECEPTOR ENZYMES ARE SPECIAL KINDS OF PROTEINS

Transmembrane receptor enzymes combine the functions of signal perception and enzyme activity in the same polypeptide. The extracellular domain, which receives the signal, is connected to the intracellular enzyme domain by a single-pass transmembrane domain (see Fig. 25-3A).

The transmembrane receptor enzymes in animal systems are of many kinds (some are even phosphatases). Mostly they are tyrosine receptor kinases (TRKs), i.e., the amino acid that is phosphorylated by ATP on perception of the signal is tyrosine. It is not quite clear how a conformational change of sufficient magnitude is brought about with a single-pass transmembrane domain, but most such receptors dimerize upon binding of the ligand and, once dimerized, the kinase domains are able to phosphorylate each other, thus amplifying the signal intracellularly. Such cross-phosphorylation between two monomers is referred to as transautophosphorylation.

In plants, all transmembrane receptor kinases identified to date with one very important exception, are serine/threonine kinases, and although dimerization is suspected it is not firmly established.

The exception is the receptor histidine kinase, which may be linked to an extracellular domain *via* two-, three-, or four-pass membrane-spanning domains and in which the amino acid that is supposed to be phosphorylated is histidine. These receptor kinases are modifications of the bacterial two-component signaling systems in which the receptor/transmitter and response regulator functions are combined in a single polypeptide (see Box 21-1 in Chapter 21). Histidine receptor kinases, once thought to be restricted to prokaryotes, are now known in increasing numbers from eukaryotes, including plants. As already shown, ETR1 and its homologues involved in ethylene perception and CK11, presumed to be involved in cytokinin per-

ception, are histidine kinases (see Chapters 21 and 24). The *WOODEN LEG* (WOL) gene in *Arabidopsis* involved in vascular morphogenesis in roots (see Chapter 3) encodes a novel type of receptor histidine kinase. The protein has a short N-terminal cytoplasmic domain followed by an extracellular domain flanked at either end by a transmembrane domain, then a cytoplasmic histidine kinase domain followed by two receiver domains. CRE1 believed to be a cytokinin receptor is identical to WOL1 (see Chapter 24).

1.1. Receptor-like Kinases (RLKs) in Plants

The number of serine/threonine receptor kinases in plants has increased rapidly since their first identification in maize in 1990. The sequenced *Arabidopsis* genome has more than 300 sequences encoding these proteins, and more sequences in other plants are being identified each year. These receptor kinases thus represent a predominant type of receptor in plants. However, the precise functions of many of them are still not known and, with few exceptions, they have not yet been shown to bind any specific ligand(s). Hence, for the time being, it is appropriate to refer to them as receptor-like kinases or RLKs.

1.2. RLKs Are Divided into Families on the Basis of Their Extracellular Domains

Based on the predicted structure of their extracellular domains, this superfamily of kinases is divided into at least 20 families, and many more subfamilies. Two common motifs are (i) the S domain and (ii) the leucine-rich repeat (LRR) (Fig. 25-13).

The S domain refers to a motif first described in certain glycoproteins that segregate with the self-incompatibility (SI) locus in genetic crosses involving *Brassica* species. SI is the recognition process by which pollen from a plant is rejected by a pistil of the same plant. The motif is characterized by an array of 10 conserved cysteine residues adjacent to the transmembrane domain and some other conserved residues considered important in proper folding of the extracellular domain.

The LRR motif is characterized by several tandem repeats of amino acid sequences rich in leucine with a conserved core sequence Leu-x-x-Leu-x-Leu-x-x-Asn-x-Leu. The repeat may be interrupted by gaps or insertions within or between repeats. LRR motifs occur in many other proteins besides transmembrane RLKs and are thought to mediate protein-protein interactions. Extracellular domains of RLKs with an LRR motif show considerable variations outside of the LRR region.

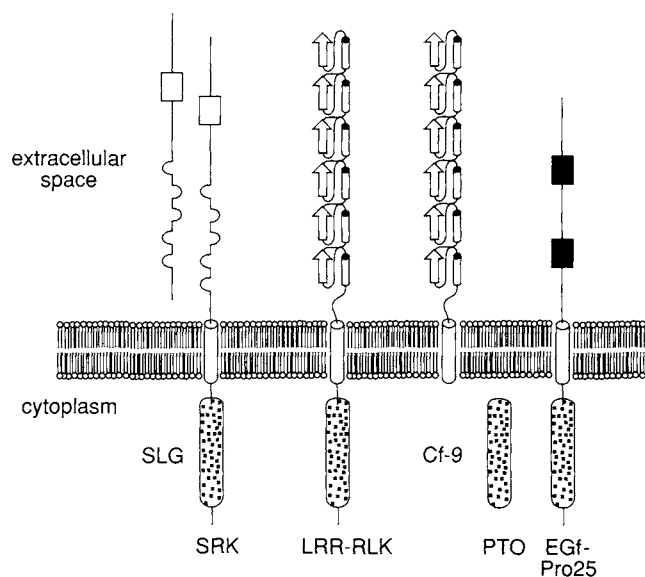


FIGURE 25-13 Schematic drawings of receptor-like kinases (RLKs) and RLK-related proteins. From left to right: a *S*-locus glycoprotein (SLG) with an *S* domain; a *S*-locus RLK (SRK1); an RLK with a leucine-rich repeat (LRR) motif; Cf-9, a protein conferring disease resistance to a leaf mold of tomato; PTO, a Ser/Thr kinase that confers resistance in tomato to a bacterial pathogen; and Pro25 with an epidermal growth factor-like domain. Adapted from Braun and Walker (1996) with permission from Elsevier Science.

1.3. RLKs Are Involved in Diverse Functions

Studies utilizing genetic crosses and/or mutants and expression patterns of mRNAs suggest that RLKs have diverse locations and cellular functions (see Table 25-2). In addition to the self-incompatibility response, they are implicated in various aspects of plant development and morphogenesis, including regulation of shoot and floral meristem size, differentiation of leaf epidermal cells, abscission, pollen development, and competence of somatic cells to form embryos. For example, *CLAVATA1* (*CLV1*) is an RLK with an LRR motif in the extracellular domain and, from mutational analysis, is known to regulate the size of both vegetative and floral shoot meristems (see Chapter 4). Other RLKs are involved in plant defense, including disease resistance in rice and wheat, and in regulating plant responses to stress tolerance against desiccation, salinity, and cold temperature. Many of these responses involve pollen–pistil, cell–cell, or plant–pathogen communication. It is conceivable that receptor kinases with an LRR repeat, *S* domain, or lectin-type domain in the extracellular region, domains thought to interact with other macromolecules, are involved in these interactions. In addition, at least two putative hormone receptors, *BRI1* in

Arabidopsis for brassinosteroid signaling and *OsTMK1* in rice for GA signaling are RLKs (see Chapter 24).

The kinase domain also seems to show substrate specificities. If RLKs are expressed as recombinant proteins in *E. coli*, they not only are autophosphorylated in the presence of ATP, their kinase domains also show preference as to whether only serine or threonine residues will be phosphorylated, or whether the majority of phosphorylated residues would be either serine or threonine. These differences in phosphorylation specificities *in vitro* suggest that the RLKs have different substrates and are involved in different functions.

1.4. RLK-Related Proteins

Proteins related to RLKs are also known. These are truncated proteins that lack one or two domains of typical RLKs (see Fig. 25-13). Examples include *S*-locus glycoprotein (SLG), which has an extracellular domain similar to that of *Brassica* SRK, but lacks the transmembrane and the kinase domains; Cf-9, a protein from tomato that confers disease resistance to a leaf mold, has an extracellular LRR repeat and a transmembrane domain, but lacks the kinase domain; and PTO, a Ser/Thr kinase that confers resistance in tomato to a bacterial pathogen, but lacks the transmembrane and extracellular domains.

Some of these truncated proteins, such as SLG, are encoded by their own genes, whereas others may be generated by a combination of alternative splicing and polyadenylation signals of the parent gene product. The function of these proteins is also unclear in many cases, although it is possible that they form heterodimers with their cognate partners and function in signal modulation.

A classic example of plant pathogen communication is provided by PTO in tomato. PTO is a truncated serine/threonine kinase that occurs in the cytoplasm. It is not known whether it dimerizes with the intracellular domain of a full-length RLK partner, but it recognizes and binds to the antivirulence factor secreted by *Pseudomonas* sp., which infects and causes leaf spot disease. Such recognition initiates a series of defense responses by the host plant.

1.5. Downstream Signaling and Small GTP-Binding Proteins

Receptor tyrosine kinases in animal systems, on receiving the signal, are autophosphorylated by ATP and start a signal transduction chain, which involves several classes of small GTP-binding proteins. These proteins, designated as small G-proteins (M_r 20–30 kDa), are GTPases and structurally similar to the

TABLE 25-2 Some Representative Receptor-like Protein kinases (RLKs) and Their Putative Functions^a

Class	Genus	RLK	Putative function	Ref.
S domain	<i>Zea</i>	ZmPK1	?	Brann and Walker (1996)
	<i>Brassica</i>	SRKs	Self-incompatibility	Brann and Walker (1996)
		SFR2	Plant defense	Pastuglia <i>et al.</i> (1997)
	<i>Arabidopsis</i>	ARK1-3	?	Brann and Walker (1996)
		RLK1	?	Brann and Walker (1996)
		RLK4	?	Brann and Walker (1996)
	<i>Oryza</i>	OsPK10	?	Brann and Walker (1996)
Leucine-rich repeat	<i>Arabidopsis</i>	HAESA	Regulates abscission	Jinn <i>et al.</i> (2000)
		ERECTA	Regulates size of siliques, petiole length	Torii <i>et al.</i> (1996)
		SERK	Expressed in cells destined to form somatic embryos in culture	Schmidt <i>et al.</i> (1997)
		CLAVATA1	Regulates shoot and floral meristem size	Clark <i>et al.</i> (1997)
		BRI1	Brassinosteroid signaling	Li and Chory (1997).
		RKF1	Affects anther and pollen development	Takahashi <i>et al.</i> (1998)
		PR5K	Plant defense	Wang <i>et al.</i> (1996)
	<i>Petunia</i>	PRK1	Anthers, pollen, and pollen tubes	Lee <i>et al.</i> (1996)
	<i>Zea</i>	CRINKLY4	Epidermal cell size	Becraft <i>et al.</i> (1996)
	<i>Oryza</i>	Xa21	Disease resistance	Song <i>et al.</i> (1995)
	<i>Triticum</i>	Others WLRK	Disease resistance	Feuillet <i>et al.</i> (1998)
	<i>Arabidopsis</i>	PRO25	Mature leaves	Brann and Walker (1996)
		—	Lectin-like domain plant defense	Herve <i>et al.</i> (1996)
	<i>Catharanthus roseus</i>	CrRLK1	?	Schulze-Muth <i>et al.</i> (1997)
	<i>Phaseolus</i>	PvRK20-1	Expressed in roots	Lange <i>et al.</i> (1999)

^aRLKs are grouped according to the two common motifs in the extracellular domains, but it should be emphasized that such grouping reflects structural similarities, not a close association in function.

G α subunit of the heterotrimeric G-proteins, but they differ in mass; they also occur as monomers. Small G-proteins transduce the signal from transmembrane receptor kinases and transmit it to one of many phosphorylating cascades. They can also trigger effector molecules (e.g., phospholipase C- γ isoform) and signal via secondary messengers.

Many different types of small G-proteins are known from animal systems and yeast. They serve diverse functions, including roles in cell proliferation, vesicle transport and secretion, cytoskeletal rearrangements, polarity establishment, and are grouped in several families within the Ras superfamily. The mammalian families include RAS, RHO, RAB, RAN, and ARF; but only Ras and Rho GTPases are considered *bona fide* signaling switches; the others are primarily involved in the regulation of trafficking of vesicles or large molecules.

The best known are the members of the RAS family, which are involved in cell proliferation. Ras is a slow GTPase, which is active when bound to GTP and inactive when bound to GDP. To bind GTP, it requires two factors: an adaptor (Grb2) and a GDP/GTP exchanger (also called Ras activator) (Fig. 25-14). The signal transduction cycle involves three steps.

- On receiving the signal, the kinase domain of the transmembrane receptor is autophosphorylated by ATP and linked to the GDP/GTP exchanger via the adaptor molecule.
- The exchanger activates Ras to replace bound GDP with GTP.
- The activated Ras transfers the phosphate group to the first of a series of protein kinases. The intrinsic phosphorylating activity in Ras is quite weak, but can be accelerated by special proteins, called

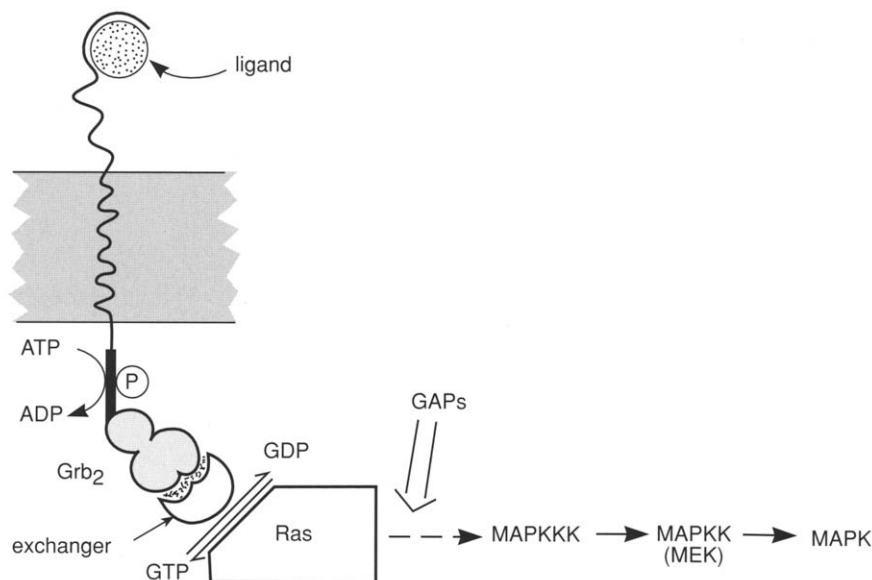


FIGURE 25-14 Tyrosine receptor kinase and signaling *via* Ras. On receiving the signal, the kinase domain of the receptor is autophosphorylated. The adaptor molecule (Grb₂) interacts with the phosphorylated receptor on the one hand and on the other binds to the exchanger. The exchanger activates Ras to exchange GDP for GTP. Some other proteins, called G-associated proteins (GAPs), augment the phosphorylating activity of Ras. Adapted from Yang (1996).

G-associated proteins (GAPs). Other proteins can slow it down.

1.6. Small G-proteins in Plants

Downstream signaling elements for plant RLKs are not known but some pieces are falling into place. First, the biochemical and immunological methods used to detect the presence of G α proteins also detect small G-proteins. They have a smaller mass and can be separated readily from their larger cousins by gel filtration; also, they do not show ADP-ribosylation by cholera toxin. Second, homology cloning using sequences from well-known small G-proteins from mammalian systems or yeast has allowed cloning of many genes in plants. Thus, orthologs of Rho and Rab are known from plants (e.g., pea, *Arabidopsis*, maize, tomato) and show varying degrees of sequence similarity with their animal or yeast counterparts. Interestingly, the Rho proteins in plants are encoded by a large family of genes, and the proteins called the Rop (Rho-related GTPase from plants) belong to a distinct subfamily of the RHO family. By contrast, orthologs of Ras, the key mediator for tyrosine kinase signaling in animals, seem to be absent in plants; at least the *Arabidopsis* genome shows no sequences related to *ras* genes. Thus, plants, while using serine/threonine kinases as receptors, also seem to use a novel type of G protein as a predominant molecular switch.

Rop GTPases in plants have been localized to different cellular locations (e.g., plasma membrane, perinuclear region, and cytosol) and implicated in a variety of signaling pathways, including tip growth in pollen tube, defense responses, synthesis of secondary wall in cotton fiber cells, vacuole development, and signaling by CLVATA1 receptor kinase (see below). Mutations in *Arabidopsis* genes encoding Rops indicate that some Rop proteins induce pleiotropic developmental phenotypes, which suggest that they act as switches in multiple and distinct signaling pathways.

There are indications that RLK signaling may be negatively regulated by other proteins, such as protein phosphatases. A protein called KAPP (for kinase-associated protein phosphatase) is known from *Arabidopsis* and maize. It is a PP2C-type phosphatase, which has been shown to specifically bind to a noncatalytic site on the autophosphorylated form of CLAVATA1 (CLV1) and some other RLKs (e.g., HAESA in *Arabidopsis*). The binding of KAPP is believed to dephosphorylate CLV1 and to inactivate it catalytically.

CLV1 is the best investigated RLK to date, and results obtained with *CLV* genes provide a possible mode of action of RLKs. Three *CLV* genes have been cloned in *Arabidopsis*. *CLV1* encodes a full-length RLK with an extracellular domain with 21 Leu-rich repeats and a cytoplasmic Ser/Thr kinase domain. *CLV2* is a smaller protein with a short cytoplasmic tail. It has an LRR in its extracellular domain, but in primary sequence is not

related to CLV1. CLV3 is a small, soluble peptide that is secreted to the cell exterior. Mutational analysis reveals that all three proteins act in the same pathway. Analysis of plant extracts indicates that CLV1 occurs in two multimeric protein complexes. The smaller CLV1 complex (185 kDa) is catalytically inactive. A functional CLV2 is required for its assembly and may form a dimer with CLV1 and be part of the complex. The larger CLV1 complex (450 kDa) includes CLV2, KAPP, and Rop GTPase. It is to this larger still inactive complex that CLV3 binds. The binding of CLV3 is thought to activate the complex and trigger the signal relay through the Rop GTPase (Fig. 25-15). The target of signaling seems to be WUCHSEL, a protein believed to regulate meristem size (see Chapter 3). Preliminary data suggest some cellular specificity for CLV proteins. While CLV3 is produced in the surface and subsurface L1 and L2 layers of the shoot meristems, CLV1 is produced in internal layers (L3) of the shoot apex. Several features in the proposed model are uncertain or unknown. They include the precise role and cellular locations of CLV2; also the identities of the proteins associated with Rop and the kinases further downstream.

CLV3 is the first known ligand for an RLK in plants; hence, some authors have started to refer to CLAVATA1 as a receptor kinase. Using a conserved stretch of amino acids in CLV3, which was known to be func-

tionally important, a search of data bases has revealed a large family of genes in *Arabidopsis* that encode proteins with the same conserved region. Likewise, another peptide, SCR, has been identified as a possible ligand for S-locus receptor kinase, SRK. SCR-type proteins are also encoded by a large gene family.

1.7 Summary of RLKs and Small G Proteins

In summary, the RLKs are an important group of receptor proteins in plants. They have three functional domains, an extracellular domain which is involved in binding to a ligand and to other proteins, a single membrane-spanning region, and a cytosolic Ser/Thr kinase domain. The RLKs are divided into families and subfamilies on the basis of structure of their extracellular domains, which indicates that they bind to different ligands and function in different signaling pathways. Moreover, *in vitro* experiments with recombinant proteins demonstrate that RLKs have different substrates and are involved in different functions. In contrast to animal systems which utilize receptor tyrosine kinases and signal via Ras-type GTPases, plants have Ser/Thr kinases and a large number of Rop GTPases, a subfamily of ROH family of GTPases, unique to plants. CLAVATA1, which is involved in determination of the shoot

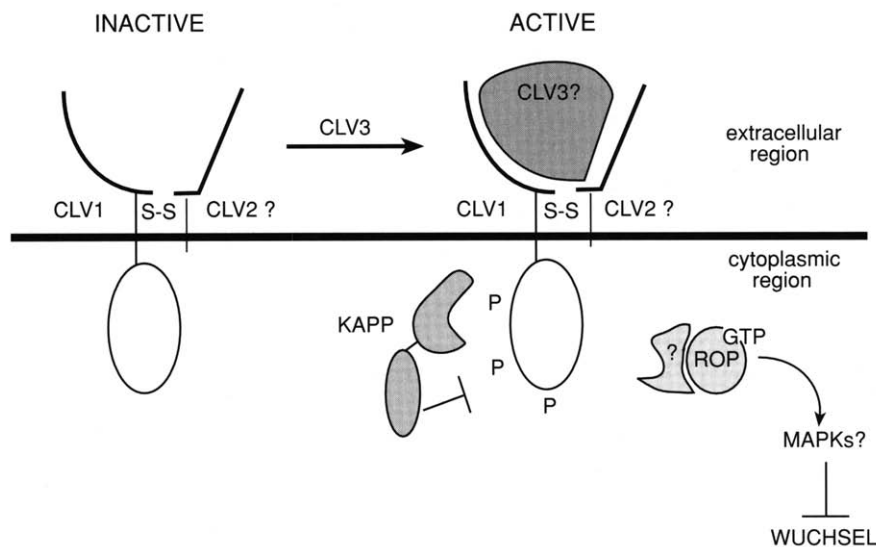


FIGURE 25-15 Model for the possible composition and function of CLV protein complexes. The inactive complex is shown as a heterodimer of CLV1 linked to CLV2 by disulfide bonds, but such linking is hypothetical. The larger complex on the right is shown with CLV1, CLV2, KAPP, and a small Rho-type GTPase (ROP). CLV1 is catalytically activated (that is, its inactivation by KAPP is removed) by binding of CLV3, a soluble protein that is secreted to extracellular space, and triggers the signal relay through ROP. The activation of ROP activates a signal cascade, perhaps *via* a MAPK pathway, to inactivate another protein, WUCHSEL, which is thought to regulate meristem size. Question marks indicate areas of uncertainty. ROP is shown separate from the phosphorylated CLV1 because possible intermediates are involved. The Rho/Rac type plant GTPases have not been shown to have a phosphoserine binding domain. Modified from Trotochaud *et al.* (1999).

and floral meristem size in *Arabidopsis*, is the best studied RLK in plants. It occurs in a multimeric complex which is kept in an inactive state by a type 2C phosphatase. The complex is activated by binding of a small peptide, CLAVATA3, and then is thought to transmit the extracellular signal via a Rop GTPase. Future studies will undoubtedly focus on elucidating the details of signaling by CLAVATA1 kinase, demonstrating the receptor functions of other RLKs, and identification of proteins associated with Rop GTPases as well as other downstream steps leading to a response.

2. MAP KINASE CASCADE

A special protein kinase cascade, known as the MAPK cascade, occurs uniquely in eukaryotic cells. Many environmental and hormonal factors activate quiescent mammalian cells to divide and are commonly referred to as mitogens (mitosis-inducing agents). Mammalian cells, arrested in G_0 and activated to divide, show a dramatic increase in the activity of a kinase, known as mitogen-activated protein kinase or MAPK. The activation of this kinase is correlated with the expression of many genes known to be involved in the entry of cells into cell cycle. Similar activation of MAP kinases and cell division-specific proteins is known for G_2 -arrested *Xenopus* and clam oocytes, which are triggered to divide by the stimulus of fertilization.

The immediate downstream targets of MAP kinases are transcription factors that bind to *cis* elements in the promoters of genes involved in cell proliferation. As expected, MAP kinases occur in cytoplasm as well as in nucleus.

The activity of a MAP kinase in quiescent cells is kept in check because MAPK is a special type of protein that needs to be phosphorylated at both a serine and a tyrosine residue, separated by one single amino acid, in order to be activated. Phosphorylating a MAPK requires a special dual-specificity protein kinase, known as MAPK kinase or MAPKK (or MEK). MEK has no other known substrates than MAPK, which provides an added measure of security against accidental or premature cell proliferation. MEK, in turn, is phosphorylated by a MAPKK kinase or MAPKKK, which is at the start of the kinase cascade (see also Box 21-1 and Fig. 21-8 in Chapter 21).

Although the MAP kinase cascade was originally discovered in the control of cell proliferation, many other MAP kinases, each with its specific MEK and MAPKKK, are known. They regulate a variety of other processes in addition to cell division and are activated by their own cognate small G-proteins, such as rho and rab. Alternatively, as mentioned before, these cas-

cades can be turned on by heterotrimeric G-proteins as well.

Thus, the MAPK cascade is a linear, signaling pathway that connects different but specific signal ligands with different end products in different organisms or cells. A single eukaryotic cell may have several dozen MAPK cascades for specific functions. For instance, in yeast cells, several homologues of MAPKK have been identified, e.g., HOG1, FUS3/KSS1, and MPK1, which, respectively, control osmosensing, pheromone-mediated mating process, and cell enlargement. These pathways are regulated in different manners: the HOG1 pathway is regulated *via* a prokaryotic two-component system involving a histidine kinase, the FUS3/KSS1 pathway by a receptor-linked heterotrimeric G protein, and MPK1 by protein kinase C. Similar heterotrimeric G-protein- and protein kinase C-mediated MAPK cascades are also found in mammals.

2.1. MAPK Cascade in Plants

Genes and cDNA clones encoding putative MAP kinases have been isolated from a number of plants (e.g., alfalfa, *Arabidopsis*, pea, tobacco). Some show >90% homology to each other, whereas others show much less, which suggests that there are different genes and isoforms of MAPKs in plants, which may operate in different signaling pathways. Some MAPKKs and at least one MAPKKK have also been cloned.

Cell divisions in tissue explants and cell cultures often require treatment with an auxin: 2,4-D, NAA, or IAA. Tobacco pith cultures have long been used to study auxin-mediated cell division (and auxin-binding proteins). A MAP kinase gene was transfected into tobacco, and pith cell cultures were used to study its expression in relation to the induction of cell division. The transcript levels were induced and enhanced by auxin and correlated with auxin-induced activation of cell division.

A MAPK homologue, which is induced by wounding [hence called wound-induced protein kinase (WIPK)] was isolated from *Arabidopsis*. WIPK is a primary response gene that is induced within minutes of wounding and does not require prior protein synthesis. Another MAPK, called salicylic acid-induced protein kinase (SIPK), has also been isolated from *Arabidopsis*. WIPK and SIPK are induced by different signals, which show that different MAPK homologues operate in the same cells.

Many studies have related induction of a MAP kinase cascade to plant responses associated with abiotic stresses, such as mechanical stimulation or wounding, and exposure to cold, drought, or high salinity. Such stresses have been shown to result in rapid, transient

activation of MAP kinases and in accumulation of specific MAPK and/or MAPKK transcripts.

2.2. MAPK Pathway in Plants May Derive Its Signal from Transmembrane Receptor Kinases of the Histidine Type

As mentioned in Chapter 21, the CTR1 protein in *Arabidopsis* is homologous to the mammalian Raf MAPKKK. While the N-terminal of CTR1 is similar to the N-terminal of Raf, it lacks the typical regulatory motif of Raf, which allows Raf to bind to the GTP-binding protein, Ras, and become activated. The immediate upstream regulator of CTR1 is unknown, but ETR1 has been proposed to regulate it directly. If so, this would link elements of the MAP kinase cascade to transmembrane receptors, which are histidine kinases. The immediate downstream elements from CTR1 are also unknown, but EIN2 and EIN3 are regulated further downstream. EIN3, as shown in Chapter 21, is a transcriptional regulator of several ethylene-induced genes.

2.3. Summary of MAPK Pathway in Plants

In summary, the major components of the MAP kinase cascade are known from plants. Several genes or cDNAs encoding MAPKs, and some encoding MAPKKs and MAPKKKs, have been cloned. The MAPK cascade seems to operate specifically in cell divisions induced by wounding or auxins, and also in many stress-related responses. However, the coupling of MAPK cascades to specific G-proteins and serine/threonine receptor kinases has not yet been possible. Plants, like yeast, show an unusual coupling of two component receptor histidine kinases with MAPK-type signaling.

It will be evident from Sections II and III in this chapter that phosphorylation plays a very important role in plant signaling. A special class of proteins are involved in stabilization of a phosphorylated protein such that it can complete its activity. These proteins known as 14-3-3 proteins are included in Box 25-3.

SECTION IV. CALCIUM AS A SECOND MESSENGER

1. CALCIUM REGULATES MANY PLANT RESPONSES

Calcium is a ubiquitous intracellular signaling molecule that mediates many growth and develop-

mental phenomena in plants as well as their responses to external stimuli. The former include tip growth in pollen tubes and root hairs, cytoplasmic streaming, vesicular secretion or exocytosis, and fixation of polarity in *Fucus* zygotes preceding asymmetric cell division (Fig. 25-16). Calcium also plays a role in hormone signaling, in gravity perception and thigmotropism, and in nongrowth phenomena, such as stomatal movements, motor responses in sun tracking, and possibly perception of temperature.

1.1. Intracellular Levels of Calcium

It is the free calcium ions in the cytosol, $[Ca^{2+}]_{cyt}$, that play a signaling role, not the calcium that may be found in bound forms such as crystals of calcium oxalate in vacuoles or bound to cell walls. However, Ca^{2+} , like oxygen, in high concentrations is extremely toxic, causing necrosis and death. It has been compared to a spark; as a brief flash it can provide a signal, but uncontrolled sparks cause a fire. Thus, a principle of crucial importance in Ca^{2+} signaling is that its cytosolic concentrations are controlled precisely. In a resting cell, the concentrations of $[Ca^{2+}]_{cyt}$ are kept low (~ 10 – 100 nM) but, when activated, can rise up to 1 – 5 μM , depending on the cell type. Such high concentrations are confined to local sites and usually are transient. In storage sites, Ca^{2+} concentrations can be much higher. In plants, the major intracellular storage sites are the vacuoles (ca. 0.1 to 10 mM) (see Fig. 25-17), followed by the endoplasmic reticulum (ER, ~ 10 μM ; this is different from animal

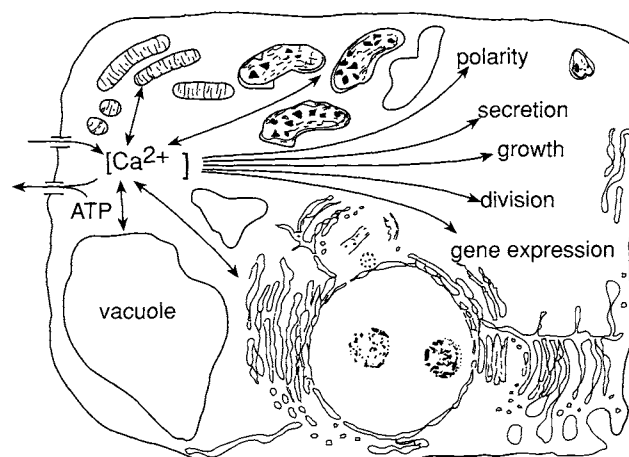


FIGURE 25-16 A cartoon showing the multiple roles of calcium in plant cells. Content of free Ca^{2+} in the cytosol is regulated by channels and pumps in plasma membrane, vacuolar membrane, endoplasmic reticulum, and in membranes surrounding mitochondria and chloroplasts. Some possible roles of cytosolic Ca^{2+} are indicated by arrows. From Trewavas and Knight (1994) with kind permission from Kluwer.

BOX 25-3 14-3-3 PROTEINS PLAY IMPORTANT ROLES IN SIGNAL TRANSDUCTION AND REGULATION OF ENZYME ACTIVITIES

1 4-3-3 PROTEINS ARE A special class of proteins that play important regulatory roles in signal transduction and regulation of enzyme activities in nearly all eukaryotic organisms. They are so named because in a study in 1967 bovine brain proteins were given numerical designations based on column fractionation and electrophoretic mobility. The name has persisted. The 14-3-3 proteins show a highly conserved central core and variable N and C termini. They occur as dimers both *in vivo* and *in vitro* and both homo- and heterodimers are formed. Each monomer has nine α -helices which form a palisade containing an amphipathic groove. The groove is lined by helices which contain the most conserved amino acid residues. Each groove is thought to accommodate part of a target protein; thus, each dimer would accommodate parts of two interacting proteins.

The 14-3-3 proteins play a critical role in pathways that are regulated by phosphorylation. They are thought to bind to the phosphorylated target such that it completes the conformational change required for its activity. This core functional characteristic is ensured by the conserved helices lining the two grooves in the dimer. Moreover, plant 14-3-3s binding to the target protein(s) requires "charging" of the 14-3-3 by Ca^{2+} via a structural reorientation of the C termini. The *Arabidopsis* isoforms possess an EF hand-like Ca^{2+} -binding motif in the C terminal region.

Each organism has several isoforms of 14-3-3 proteins which are encoded by members of a gene family. The proteins occur in chloroplasts, mitochondria, nucleus, and cytoplasm, which suggests some functional diversity. The number of isoforms coupled with the possibility of heterodimerization offers a large variety of combinations to accommodate many different kinds of target proteins. For example, in plants, 14-3-3s are believed to participate in binding of transcription factors to *cis*-sequences in promoters of certain genes in maize and *Arabidopsis*; serve as a nuclear matrix endonuclease in pumpkin; and associate with Raf activity, complex with cdc25 phosphatases, and act as an essential component in the functioning of an H^+ -ATPase regulated by the fungal toxin, fusicoccin. They are involved in regulation of the light- and substrate-regulated metabolic enzymes in nitrogen and carbon assimilation (e.g., nitrate reductase and sucrose phosphate synthase, respectively) and in starch synthesis (e.g., starch synthase) to name a few.

cells, where the largest reservoir of intracellular calcium is in the ER). Plastids and mitochondria have limited amounts. The major extracellular storage site is the cell wall ($\sim 1 \text{ mM}$) where most of Ca^{2+} occurs bound to pectins.

1.2. How Do We Measure Intracellular Calcium Concentrations?

Intracellular levels of calcium $[\text{Ca}^{2+}]_{\text{cyt}}$ are measured using Ca^{2+} -sensitive dyes, especially those that fluoresce in visible light after excitation at a lower wavelength (usually UV, 340 or 380 nm). These include nonratiometric and ratiometric dyes such as Calcium Green-1 and Fura-2. These dyes are typically microinjected and the cells are examined using an inverted epifluorescence microscope equipped with a fluorescence emission detector. The signal is amplified, digitized, and processed in different ways to provide a good

temporal (fluorescence ratio photometry) or a temporal as well as spatial separation (fluorescence ratio imaging) of $[\text{Ca}^{2+}]_{\text{cyt}}$. In more recent years, confocal laser scanning microscopy (CLSM) has become the method of choice (see Box 2-4 in Chapter 2).

1.3. Calcium Channels and Pumps

The concentrations of $[\text{Ca}^{2+}]_{\text{cyt}}$ are regulated by channels and pumps. The movement of calcium downhill from extra- or intracellular sites into the cytoplasm is accomplished *via* a variety of channel proteins, e.g., voltage-activated channels, stretch-activated channels, and ligand-activated channels. The entry of Ca^{2+} into cytoplasm from external sources involves voltage- or stretch-activated channels located in the plasma membrane. Its release from intracellular storage sites occurs via both voltage- and ligand-activated channels. Ligand-activated channels specific to calcium are of

two main types: those activated by binding of IP_3 and those activated by binding of cyclic adenosine 5'-diphosphoribose (cADPR). The receptors for IP_3 or cADPR (the latter resemble ryanodine receptors on the ER in animal cells) are tetrameric channel proteins (for IP_3 receptor, see Section II,1.2). Their presence in plants is deduced (see below), but they are thought to be located on the ER and/or vacuolar membranes. The differential activation of Ca^{2+} entry or release channels allows cells to respond to a range of diverse

stimuli and produce Ca^{2+} signals that are tissue specific. Removal of Ca^{2+} from the cytosol and its accumulation in organelles, such as ER and vacuoles, and the cell wall occurs against a concentration gradient and requires energy. It utilizes pumps (ATPases) or H^+ motive force to drive Ca^{2+} movement uphill (see Fig. 25-17). Cytosolic Ca^{2+} may also be removed by various types of Ca^{2+} -binding proteins (e.g., cytoskeletal proteins, calmodulin, or Ca^{2+} -dependent protein kinases).

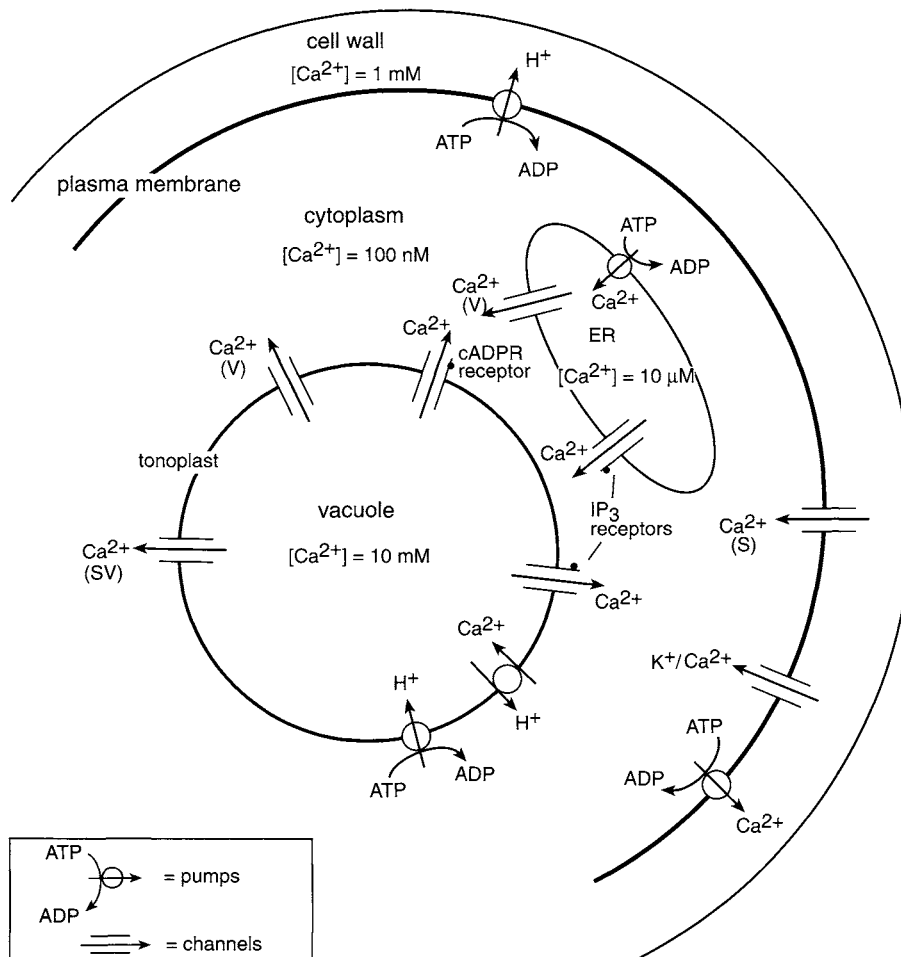


FIGURE 25-17 Calcium channels and pumps and intra- and extracellular concentrations of calcium in a typical plant cell. The outward Ca^{2+} transport across the plasma membrane is mediated by a Ca^{2+} -ATPase coupled to a $\text{Ca}^{2+}/\text{NH}_4^+$ antiporter. Accumulation in the ER is mediated by a Ca^{2+} -ATPase and in the vacuole by a tonoplast-associated H^+ -ATPase, which pumps protons into the cytoplasm for exchange of Ca^{2+} . Channels include voltage activated (V) and stretch activated (S) on the plasma membrane. They are utilized for entry of Ca^{2+} into the cells from the outside. The vacuolar membrane has two types of voltage-activated channels. The V channel is activated by membrane hyperpolarization, whereas the SV (slowly activating vacuolar) channel is activated by membrane depolarization, and also by an increase in $[\text{Ca}^{2+}]_{\text{cyt}}$. The SV channel is believed to participate in CICR (see text). IP_3 and cADPR receptor channels (identified by a dot) are shown located on the vacuolar membranes; those for IP_3 are on the ER membranes. These channels are activated upon perception of the ligands, IP_3 or cADPR, respectively, and release Ca^{2+} into the cytoplasm. Approximate concentrations of Ca^{2+} in various cellular compartments are indicated. Modified from Sanders *et al.* (1999).

1.4. Elements of Ca^{2+} Signaling

How does a simple ion like Ca^{2+} mediate diverse physiological or biochemical responses? The answer lies in the fact that Ca^{2+} signals can be enormously versatile. Much of the information presented here comes from animal cells, although it is likely that similar processes occur in plants. On receiving the appropriate signal, the voltage- or receptor-activated channels open and release Ca^{2+} into the cytosol. The channels only open for a short time, however; thus, they introduce brief pulses of Ca^{2+} that form a small plume around the mouth of the channel before diffusing into the cytoplasm (Fig. 25-18). In animal systems, these plumes have been visualized using confocal microscopy of living cells, and a variety of cell types have been shown to produce them. They are designated "elementary" Ca^{2+} signals and are considered as the basic ingredient of Ca^{2+} signaling. For example, a transient opening of a voltage- or receptor-activated channel may raise the $[\text{Ca}^{2+}]_{\text{cyt}}$ to 300–400 nM in its vicinity (within 1–6 μm), which is sufficient to allow a highly specific calcium signal to be perceived by a target protein located within the range. Such a localized signaling may open ion channels or promote exocytosis. The ascending curve of a Ca^{2+} transient is thought to represent Ca^{2+} uptake from external medium or internal reserves; the downward curve its extrusion out of the cell or sequestration in internal storage compartments.

IP_3 and ryanodine receptor-activated channels are sensitive to low levels of cytosolic Ca^{2+} , even though they open only in response to IP_3 or cADPR. The stimulatory effect of cytosolic Ca^{2+} means that these receptors display the phenomenon of Ca^{2+} -induced

Ca^{2+} release (CICR). In plant cells, SV channels on the vacuolar membrane are also subject to CICR. CICR is an autocatalytic process of fundamental importance in which an elementary Ca^{2+} signal recruits another channel or a cluster of channels to give rise to Ca^{2+} spikes (oscillations) or waves. In a Ca^{2+} spike, the elementary signal diffuses and recruits more channels locally, thus amplifying the signal and providing the characteristic spike. An elementary Ca^{2+} signal originating in one region of the cell may also be amplified and propagated through the cell. Such propagation requires a coordinated recruitment of new clusters of channels in a progressive manner such that the Ca^{2+} released from a channel diffuses to a new channel and activates it. Diffusion may or may not be coupled with the retrieval of $[\text{Ca}^{2+}]_{\text{cyt}}$ into storage sites or by Ca^{2+} -binding proteins. This progressive release of Ca^{2+} (and its diffusion/retrieval) sets up a wave that spreads in a regenerative manner through the cell (Fig. 25-19). Such waves travel relatively slowly (about 10–100 $\mu\text{m}/\text{sec}$), but can spread from one part of the cell to another or to a neighboring cell *via* plasmodesmata. Propagation of a Ca^{2+} wave can provide global signaling.

Ca^{2+} spikes (oscillations) and waves are two forms in which the Ca^{2+} signal is frequently presented to the cytoplasm of a cell. Oscillations in one location, like a stationary beacon, provide signaling information in several dimensions: amplitude (or strength, i.e., the amount of Ca^{2+} released at each opening), frequency (or period, i.e., the interval between successive openings), and duration (i.e., the total time over which signaling continues). A wave carries more information. Analogous to a moving beacon or a firefly, it has the added content of direction. Each wave has a point of origin and a point of termination, which is

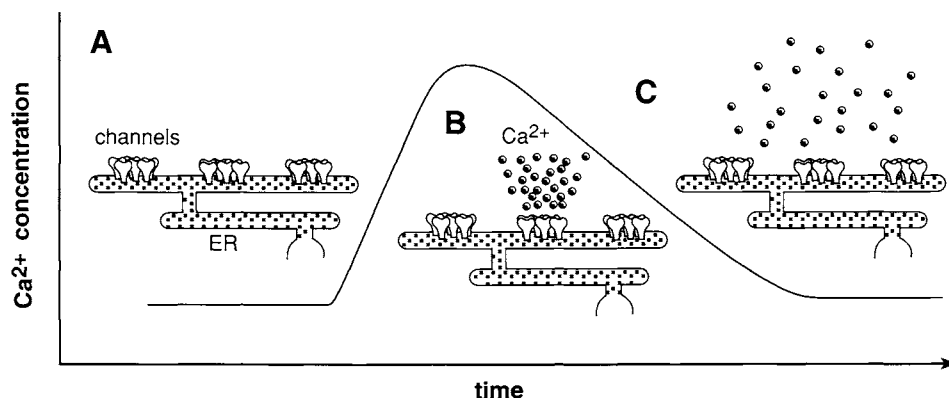


FIGURE 25-18 A typical elementary event resulting from the localized release of Ca^{2+} from channels (ryanodine or IP_3 receptors) located in the endoplasmic reticulum (ER) of an animal cell. (A) Quiescent state. (B) A group of channels open to form a plume (also called "spark" or "puff," depending on the type of channel). (C) The channels shut and Ca^{2+} diffuses into the cytoplasm. From Berridge *et al.* (1999) with permission from Elsevier Science.

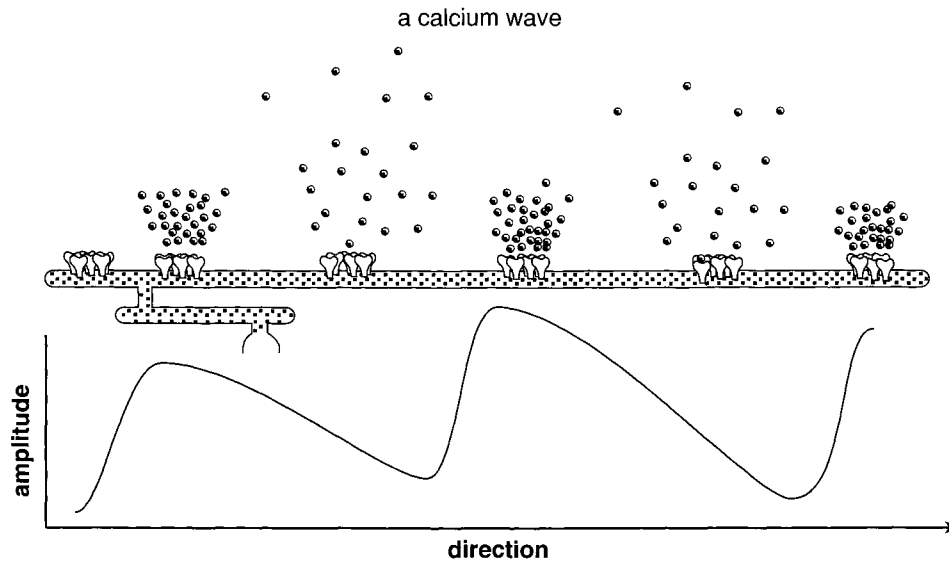


FIGURE 25-19 A hypothetical scheme showing the generation of global Ca^{2+} signals. An elementary Ca^{2+} release recruits other groups of channels in a progressive manner. The release events alternate with the diffusion of Ca^{2+} and/or retrieval by Ca^{2+} pumps or intracellular-binding proteins. The combination of release and diffusion/retrieval sets up a Ca^{2+} wave, which provides global signaling. Adapted from Berridge *et al.* (1999) with permission from Elsevier Science.

usually the site of physiological response. Ca^{2+} waves are thought to be the form in which Ca^{2+} signaling information enters the nucleus. $[\text{Ca}^{2+}]_{\text{cyt}}$ gradients are the third type of Ca^{2+} signal; they are the least versatile of the three, but can provide steady long-term signaling as in tips of growing pollen tubes or root hairs,

1.5. Calcium Signaling in Plants

A large number of responses mediated by hormones, environmental factors (e.g., touch, wind, light), and stress due to biotic or abiotic agents are known to induce changes in levels of $[\text{Ca}^{2+}]_{\text{cyt}}$ (see Box 25-4). Changes are mostly an increase in free

Ca^{2+} levels, although decreases are also reported in some cases. Thus, gibberellin-induced synthesis and secretion of α -amylase in cereal aleurone are preceded by an increase in levels of $[\text{Ca}^{2+}]_{\text{cyt}}$, whereas ABA inhibits such an increase. In guard cells, ABA induces an increase in the concentration of $[\text{Ca}^{2+}]_{\text{cyt}}$, leading to a loss of turgor and stomatal pore closure (see Chapter 23). Exposure of plants to ozone triggers a stress response and expression of a glutathione *S*-transferase (*GST*) gene, which is coupled to a rise in $[\text{Ca}^{2+}]_{\text{cyt}}$. Plants are sensitive to mechanical stimulation, hypotonic shock, and fungal elicitors, and transient and apparently large increases in $[\text{Ca}^{2+}]_{\text{cyt}}$ occur in response.

BOX 25-4 AEQUORIN CAN BE USED TO DISPLAY CALCIUM TRANSIENTS

AEQUORIN IS A CALCIUM-SENSITIVE luminiscent protein from the jelly fish, *Aequorea victoria*. In the jellyfish, aequorin occurs in certain cells. When the fish is threatened with predation, Ca^{2+} levels in these cells are increased and the protein luminesces, thus inhibiting predation. Aequorin is a 21-kDa protein, composed of an apoprotein and an imidazolopyrazine luminophore, coelenterazine. On interaction with Ca^{2+} , coelenterazine is oxidized to coelenteramide and light is emitted.

Anthony Trewavas and associates at Edinburgh University have used the gene for aequorin apoprotein to transform tobacco plants (*Nicotiana plumbaginifolia*). The transformed plants express aequorin and, when dipped in a solution of coelenterazine, form actively reporting aequorin. Since aequorin is a soluble protein, the luminescent plants directly report fluxes in $[\text{Ca}^{2+}]_{\text{cyt}}$ by changes in luminosity (Fig. 25-20).

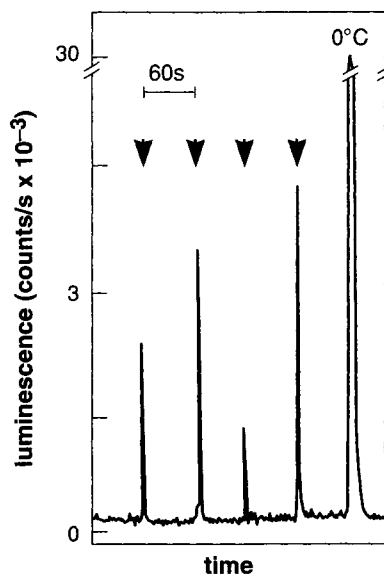


FIGURE 25-20 Changes in cytosolic $[Ca^{2+}]_{cyt}$ as measured by aequorin and luminophore. One-week-old tobacco seedlings transformed with aequorin were placed in a luminometer and touched gently with a fine wire once every minute for 4 min (indicated by arrows). After this stimulation the seedlings were irrigated with ice-cold water (extreme right). Changes in $[Ca^{2+}]_{cyt}$, represented by changes in luminosity, appear as spikes. From Trewavas and Knight (1994) with kind permission from Kluwer.

Aequorin has been very useful in demonstrating calcium fluxes in living plant cells and the role of calcium signaling in various plant responses, but has limitations, the chief being the limited number of times the aequorin inside the cell can be used. Apparently, transgenic aequorin requires constant turnover and, after some time, the plant cells stop making it. Also, the kinetics of Ca^{2+} changes as measured by aequorin differ from those measured using fluorescent Ca^{2+} sensitive dyes.

Although IP_3 and cADPR receptors have not yet been demonstrated in plants, some of the strongest evidence for a role of Ca^{2+} in plant signaling, specifically ABA signaling, is provided by the injection of secondary messengers, IP_3 or cADPR. For example, release of caged IP_3 inside guard cells leads to a rise in free Ca^{2+} and eventual closure of the stomatal pore without treatment with exogenous ABA. Similarly, guard cells injected with cADPR show a release of Ca^{2+} and a decrease in stomatal pore size without ABA treatment; also, vacuoles isolated from guard cell protoplasts release calcium when treated with cADPR. *RD29A* and *KIN2* are ABA- and cold temperature-responsive genes in *Arabidopsis* (see Chapter 16). Tomato plants transformed with *RD29A::GUS* or *KIN2::GUS* constructs show expression of the reporter gene when micro-injected with cADPR in the absence of exogenous ABA.

1.6. Oscillations and Waves Provide Unique Signatures for a Diversity of Signaling Needs

The various responses thought to be mediated via calcium have been difficult to explain simply on the basis of fluctuations in the levels of cytosolic calcium. For example, both auxin and ABA have been proposed to regulate guard cell behavior *via* changes in $[Ca^{2+}]_{cyt}$, but have opposite effects on stomatal aperture. However, as explained earlier, Ca^{2+} oscillations and waves can be very versatile and diverse and carry sufficient unique information to provide specific signaling. For example, both the frequency (pulsing rate) and the amplitude (amount of Ca^{2+} released by a single or a cluster of channels at each pulse) of oscillations provide a unique signal, which has been referred to as the "calcium signature" (Fig. 25-21).

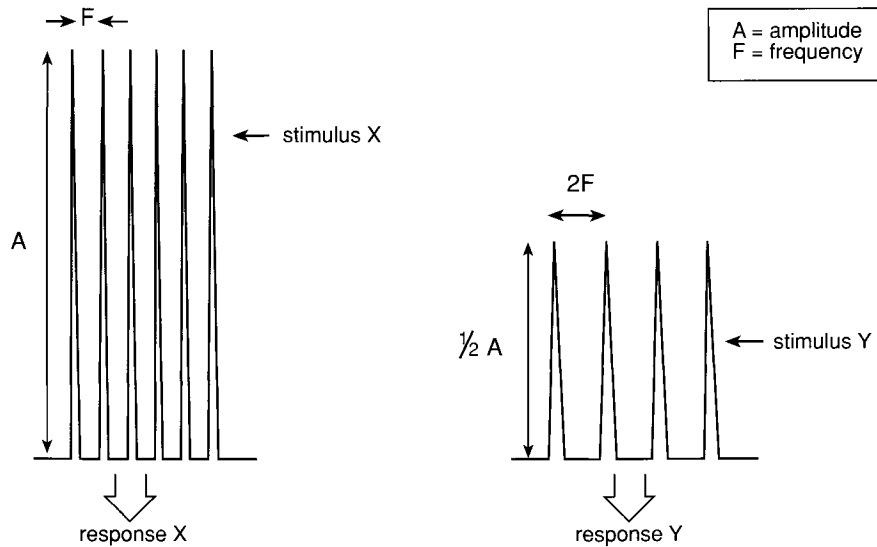


FIGURE 25-21 Transient changes in $[Ca^{2+}]_{cyt}$, plotted in the form of frequency (pulse) and amplitude (amount of calcium released), provide unique signaling information known as the “calcium signature.” Stimulus X and stimulus Y elicit Ca^{2+} spikes, which differ in both amplitude and frequency. From McAnish and Hetherington (1998) with permission from Elsevier Science.

In Chapter 23, it was mentioned that although ABA causes stomatal closure and a rise in cytosolic calcium levels in guard cells preceding stomatal closure, there is no clear correlation between the provision of exogenous ABA and the rise in cytosolic Ca^{2+} . Some of this lack of correlation is undoubtedly due to the inherent variability in biological systems and to the physiological and developmental states of guard cells. Recently, this correlation was shown to operate in guard cells of *Commelina* using the concept of calcium signature. Epidermal strips placed in buffer solution with a noninducing concentration of ABA exhibited a slight reduction in stomatal pore aperture, but when the ABA concentration was raised to $1 \mu M$, there was an immediate change resulting in “fully closed” pores. The change in ABA concentration also induced a simultaneous change in the period and amplitude of oscillations in cytosolic $[Ca^{2+}]_{cyt}$, thus coupling the calcium signature to the initiating signal, ABA (Fig. 25-22).

The *DE-ETIOLATED3* (*DET3*) gene in *Arabidopsis* encodes the C subunit of the vacuolar H^+ -ATPase, which plays a role in the solute uptake in vacuoles and consequently water uptake and plant growth. Mutants defective in the gene are impaired in water uptake and are dwarfs. Since stomatal behavior also depends on water uptake and release by guard cell vacuoles, *det3* mutants were used to study Ca^{2+} signaling in guard cells. In wild-type guard cells, ABA, oxidative stress, cold, and external calcium elicited oscillations in $[Ca^{2+}]_{cyt}$ of differing amplitudes and

frequencies and caused stomatal closure. In *det3* mutant guard cells, external calcium and oxidative stress elicited prolonged calcium increases, which did not oscillate and stomatal closure was abolished. In contrast, cold and ABA elicited $[Ca^{2+}]_{cyt}$ oscillations in *det3* and stomatal closure progressed normally. Moreover, in the *det3* mutant, artificially inducing calcium oscillations in guard cells rescued stomatal closure. These data provide compelling genetic evidence that oscillations in $[Ca^{2+}]_{cyt}$, i.e., both a rise and a decline, are important for calcium signaling. Second, they demonstrate that pathways for calcium signaling for stomatal closure are different for cold and ABA from those used by oxidative stress and external calcium.

Ca^{2+} waves and elemental Ca^{2+} release in plant cells have been recorded in the embryos of marine brown alga, *Fucus*. In their natural intertidal habitat, these embryos are subject to dramatic fluctuations in external osmotic conditions, varying between extreme hypo- and hypersalinity. Two-celled embryos (with a rhizoid cell and a thallus cell) subjected to hypoosmotic shock show an explosive Ca^{2+} increase, and the signal is propagated bidirectionally at approximately $20 \mu m/s$. Scans of intracellular Ca^{2+} reveal nonuniform Ca^{2+} elevations in highly discrete domains in both the rhizoid apex and the perinuclear space. Individual events are spatially confined and last for 15–30 ms. Since *Fucus* rhizoids, like pollen tubes, do not have large vacuoles, the Ca^{2+} release probably involved channels located on ER storage sites.

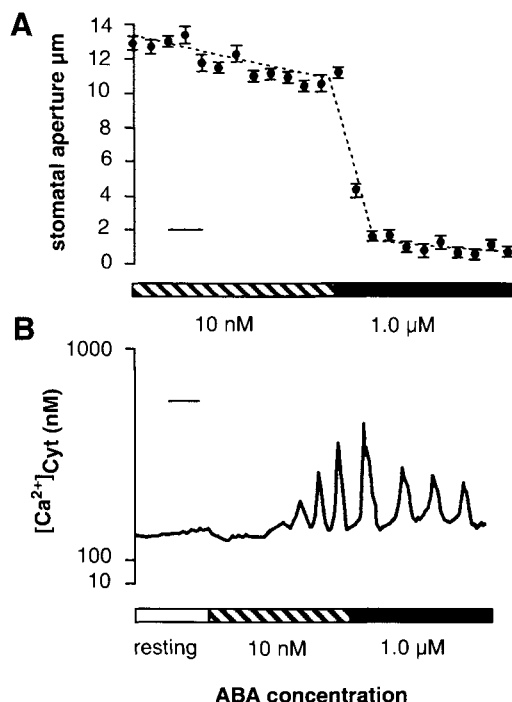


FIGURE 25-22 ABA-induced stomatal closure and patterns of oscillations in $[\text{Ca}^{2+}]_{\text{cyt}}$ in *Commelina* leaf epidermal strips. (A) Epidermal strips were allowed to equilibrate in buffer for 3 h before transfer to buffer plus 10 nM ABA (hatched bar). After 1 h, the ABA concentration was increased to 1.0 μM (solid bar). Stomatal apertures were recorded every 5 min throughout the experiment. Values are the means of 120 measurements \pm SEM. Bar: 10 min. (B) Fura-2-loaded guard cells of open stomata ($> 6 \mu\text{m}$ pore aperture) were perfused with buffer, and the resting $[\text{Ca}^{2+}]_{\text{cyt}}$ was determined (open bar). Subsequently, guard cells were perfused with buffer plus 10 nM ABA (hatched bar). After the characteristic oscillations in $[\text{Ca}^{2+}]_{\text{cyt}}$ were established, the ABA concentration was increased to 1.0 μM . A representative trace is shown. Bar: 15 min. From Staxén *et al.* (1999) with permission.

1.7. Downstream Signaling

The downstream targets of Ca^{2+} signaling are thought to be ion channels, Ca^{2+} /CaM-activated protein kinases or phosphatases, calcium-dependent protein kinases, and gene expression (see earlier discussion). Many cytoskeletal proteins bind Ca^{2+} , and such binding may mediate cytoskeletal rearrangements associated with cell division and cell growth. We also know that oscillations in cytosolic calcium are necessary for stomatal pore closing. However, the exact manner in which the calcium signals are linked to downstream events is still mostly unknown.

2. SECTION SUMMARY

In resting cells, levels of cytosolic free calcium, $[\text{Ca}^{2+}]_{\text{cyt}}$, are maintained at relatively low levels, but,

when activated, these levels rise sharply, although transiently. The changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ are brought about by the combined activities of a variety of channels and pumps. The channels facilitate the entry of Ca^{2+} from external sites or release from internal stores, whereas the pumps and various Ca^{2+} -binding proteins sequester and, thus, reduce the levels of cytosolic Ca^{2+} . The transient release of Ca^{2+} from a channel creates the elementary Ca^{2+} signal, which can lead to Ca^{2+} spikes (oscillations), Ca^{2+} waves, or Ca^{2+} gradients. Oscillations and waves carry more information than gradients and are frequently the manner in which the Ca^{2+} signal is presented to the cytoplasm. The specific signaling information carried in a series of oscillations, or a wave, may provide the specificity needed to link a calcium signal to a biological response. Although many pieces of calcium signaling, such as the release of Ca^{2+} from intracellular sources, the elements of Ca^{2+} signaling, and demonstration that a specific response requires a specific calcium signature, are falling into place, there is still little information on the linking of the Ca^{2+} signal to downstream targets.

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SECTION I. GPCRS AND HETEROTRIMERIC G. PROTEINS

3. 7 TM receptors and Heterotrimeric G-Proteins in Plants

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4

Hormone-Binding Assays and Protein Purification

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1. HORMONE-BINDING ASSAYS

One approach to isolating a receptor is to tag it with a labeled hormone. These assays, called hormone-binding assays, require radiolabeled hormones of high specific activity and purity. The labeled hormones are used for *in vivo* or *in vitro*-binding assays. *In vitro* assays have the advantage that the binding fractions can be purified and the receptor protein isolated. In actual practice, this has proven very difficult for most hormones and, hence, the more general term, hormone-binding protein, is used.

1.1. Hormone-Binding Studies Require Hormones of High Radiospecificity

Since hormones are active in small doses, 10^{-7} to 10^{-10} M (or 100 nM to 100 pM) range, and the receptor molecules, assuming one to one binding, occur in similar amounts in target tissues, one requires labeled

hormones with very high specific radioactivity (usually $> 10 \text{ Ci mmol}^{-1}$) to detect their presence amid a mass of other proteins (or macromolecules). Such high specific radioactivity is usually possible only with ^3H -labeled compounds although ^{14}C -labeled hormones have been used). It is also important that the radiolabeled hormone be chemically as pure as possible. For instance, a 100 nM solution of a radiolabeled hormone with a 1% contamination still has the contaminant(s) at 1 nM concentration, an intolerable level. These requirements for high specific radioactivity and purity can be relaxed if the candidate protein has been purified or if it occurs in a nearly pure state.

1.2. Protocols for Binding Assays

Binding assays can be done *in vivo*, but for precise quantitative data, *in vitro* assays are required. An *in vivo* assay requires a target tissue, i.e., protoplasts or tissue slices from the target zone; the tissue is incubated with the labeled hormone and, after a suitable incubation period, the free hormone is separated from that bound to macromolecules in the tissue by quick rinsing or blotting or some other suitable protocol. The radioactivity bound in the sample is solubilized and counted in a scintillation counter.

In vitro assays require a tissue extract or partially purified protein fraction, which is incubated with the labeled hormone. Equilibrium dialysis, as for ligand binding assays (Fig. A4-1), can be used, but has not proven fruitful for most plant hormones, partly because it is slow and proteins can degrade and partly because of low abundance of receptor proteins.

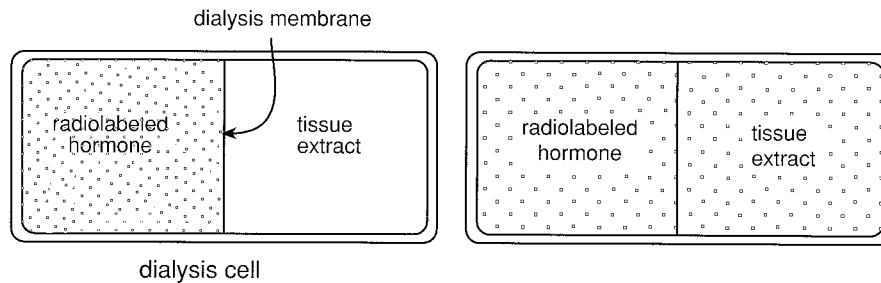


FIGURE A4-1 Principle of equilibrium dialysis. A dialysis apparatus has two compartments separated by a membrane. The labeled hormone is placed in one compartment, while the protein is placed in the other compartment. The membrane allows the passage of small molecules, such as the ligand, but restricts the passage of larger molecules above a certain molecular mass (e.g., 5000 kDa). After incubation for some time, an equilibrium is reached. Samples from the two compartments are taken and radioactivity is counted. Because of binding to the protein, the total radioactivity in the compartment with the protein is higher than in the other compartment. The difference gives the amount of the hormone bound.

Other methods involve incubation of the extract with the labeled hormone, followed by separation of the hormone fraction, which is bound to the protein extract from that which is free (Fig. A4-2). For membrane-bound receptors, the bound hormone, along with membrane fraction, can be filtered out or centrifuged and pelleted, rinsed appropriately, and counted. For soluble receptors, the bound activity may be separated from the free hormone by filtration, which keeps the protein fraction on the filter, or precipitated by ammonium sulfate and centrifuged with gentle washing.

1.3. Criteria for Receptor-Type Binding

Several criteria are used to distinguish binding of radiolabeled hormone to a putative receptor as opposed to spurious or nonspecific binding to other macromolecules.

i. **Saturability.** Since any target tissue or tissue extract is *a priori* assumed to have a defined number of receptor molecules, a saturation of binding sites should be reached. For *in vivo* assays particularly, but also for *in vitro* assays, metabolism of the hormone during the incubation period is a problem to be reckoned with. If metabolism of the hormone to inactive forms is occurring, the uptake may continue unimpeded and there may be no apparent saturation (Fig. A4-3A). Metabolism can be curtailed by performing the binding assays at close to 0°C—a low temperature reduces or eliminates metabolism without significantly affecting hormone binding. However, some hormone-binding assays have been performed at 25°C. Alternatively, and this is particularly true for auxin-binding studies, synthetic auxins may be used, which are not metabolized readily by plant tissues or extracts.

ii. **Specificity.** Binding should be specific to biologically active hormones or analogs; less active analogs should bind to a lesser extent, and inactive analogs or derivatives should not bind.

iii. **Binding is exchangeable.** Binding to a receptor is believed to be noncovalent and exchangeable. Therefore, it can be competed for by biologically active analogs, or exchanged with them, in proportion to their biological activity. An analog that has about the same biological activity as the hormone in question, used at the same concentration as the hormone, will displace the bound hormone by 50%; another analog that has lesser activity will displace the bound hormone to a lesser extent (Fig. A4-3B).

iv. **Binding is of high affinity.** The concentration of the hormone at which one-half available receptor sites

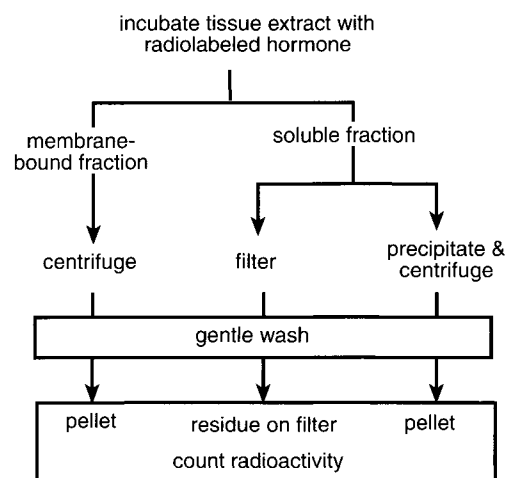


FIGURE A4-2 Protocols for hormone binding to membrane fractions or soluble proteins. The filter used for soluble fractions is either charged (and hence retains the protein) or excludes proteins above a defined size.

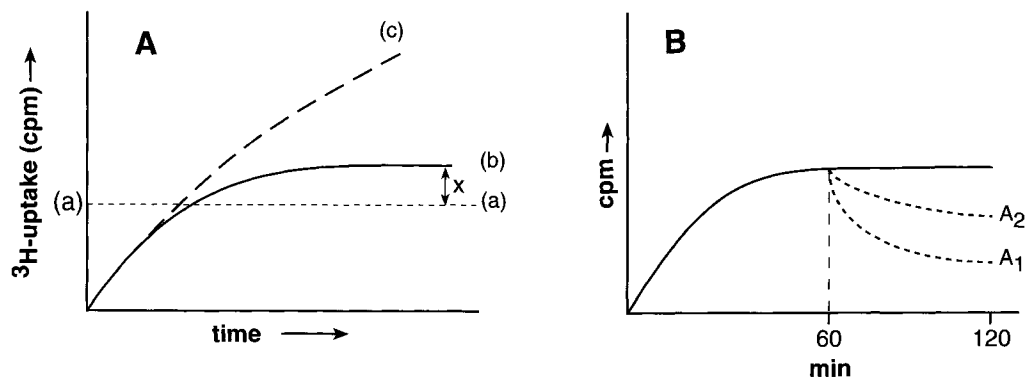


FIGURE A4-3 Binding assay showing saturation and exchangeability. (A) Saturation of hormone uptake. Line a-a shows hormone concentration in the ambient medium. The saturability of binding sites is equated with the saturation of uptake (curve b). This is strictly true only if the hormone taken up is not converted to other molecular species by metabolism. (c) Plot of uptake in the presence of metabolism. x represents cpm bound to macromolecular components. (B) Exchangeability is demonstrated by adding a nonradioactive analog after the saturation of uptake has been reached. In the illustration, analog A_1 is more active than analog A_2 . Both are used at the same concentration, but A_1 displaces more labeled hormone than A_2 .

are saturated is referred to as the dissociation constant and is denoted by the symbol K_D . A binding is considered to be of high affinity if the K_D is 100 nM or less. Enzymes usually have K_M values (equivalent to K_D) values of 1.0 μM or higher for their substrates. Moreover, the calculated K_D should bear some reasonable relationship to dose-response curves or hormonal concentrations, which elicit physiological responses.

1.4. Specific vs Nonspecific Binding

In actual practice, not only the receptor but many other proteins, membranes, cell wall fractions, and so on bind to the radiolabeled hormone. Most of this binding is nonspecific and covalent. To reduce or eliminate this nonspecific component from calculations, the plant tissue or tissue extract is incubated with the labeled hormone, which binds to both specific and nonspecific sites. The sample is divided into two, and to one-half, an excess (about a 1000 \times excess) of cold or nonradioactive hormone is added. The excess of cold hormone displaces the labeled hormone from specific sites (because they were bound noncovalently). The difference in counts per minute (cpms) between the two counts—one without and the other with the cold hormone—is used to calculate the cpms specifically bound (Fig. A4-4). The cpms are converted to disintegrations per minute (dpm). Knowing the dpm specifically bound and the specific radioactivity of the labeled hormone, one can compute the actual moles of hormone bound. If the amount of protein used is known, the bound activity can be expressed as moles of hormone bound per milligram of protein.

1.5. Calculation of Binding Affinity and Number of Binding Sites

For calculation of binding affinity and number of binding sites, a series of incubations with the labeled hormone at a reasonably low concentration ($\sim 5 \times 10^{-8} \text{ M}$), plus cold hormone over a range of concentrations, from 0 to saturating concentration ($\sim 10^{-4} \text{ M}$), are made. (Alternatively, the radiolabeled hormone may be used at a range of concentrations, while keeping the unlabeled hormone at the same level.) The amount of labeled hormone specifically bound is calculated for each incubation. Data are plotted by various methods. One such method, known after Scatchard (1949), plots the ratio of labeled hormone

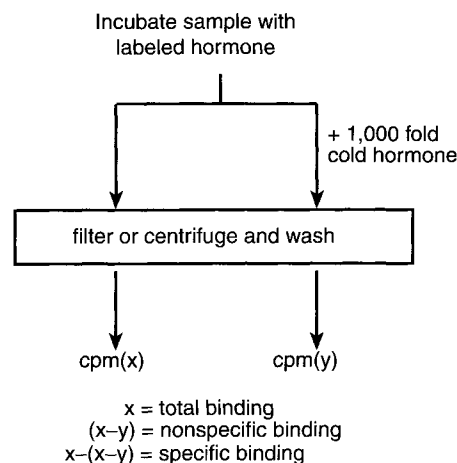


FIGURE A4-4 Protocol for determination of specific binding. See text for details.

specifically bound and the total labeled hormone supplied along the y axis and the labeled hormone bound per milligram protein along the x axis. Scatchard plots are used to calculate the K_D , or the affinity, of the receptor for the particular hormone, as well as the number of binding sites (n) in moles \cdot g $^{-1}$ protein or fresh weight.

Note: In actuality cpm's bound specifically still include binding to nonreceptor components. To separate these, purer protein fractions need to be used in binding assays and a discrimination made on the basis of K_D —lower the numerical value of K_D and higher the affinity, the greater the chances that one is dealing with the receptor protein.

2. PURIFICATION OF RECEPTOR PROTEINS

2.1. Chromatographic Methods

If *in vitro* binding can be obtained, then it is possible to fractionate the bound fractions and to try to purify

the receptor protein. However, this is very difficult in practice, and conventional biochemical techniques, including size-exclusion and ion-exchange chromatography coupled with binding assays, have not proven successful for two main reasons: (i) the receptor proteins occur in small amounts and to separate them from large masses of other proteins is difficult and (ii) the binding assays must be precise enough to distinguish between binding to the receptor and to other, nonreceptor proteins that may also show specific and exchangeable binding, but differ in affinity or K_D of binding. Accordingly, affinity and immunoaffinity chromatography have been used as a first-step purification.

Affinity purification utilizes the affinity of a protein for its ligand. The ligand, either directly or *via* a conjugated protein, is bound covalently to a substrate, such as a Sepharose bead (Fig. A4-5A). The crude extract is passed through the affinity column where the protein of interest gets bound to the ligand, whereas the rest of the extract goes through. The bound protein is subsequently eluted with salt or a solution of a hormone of higher affinity.

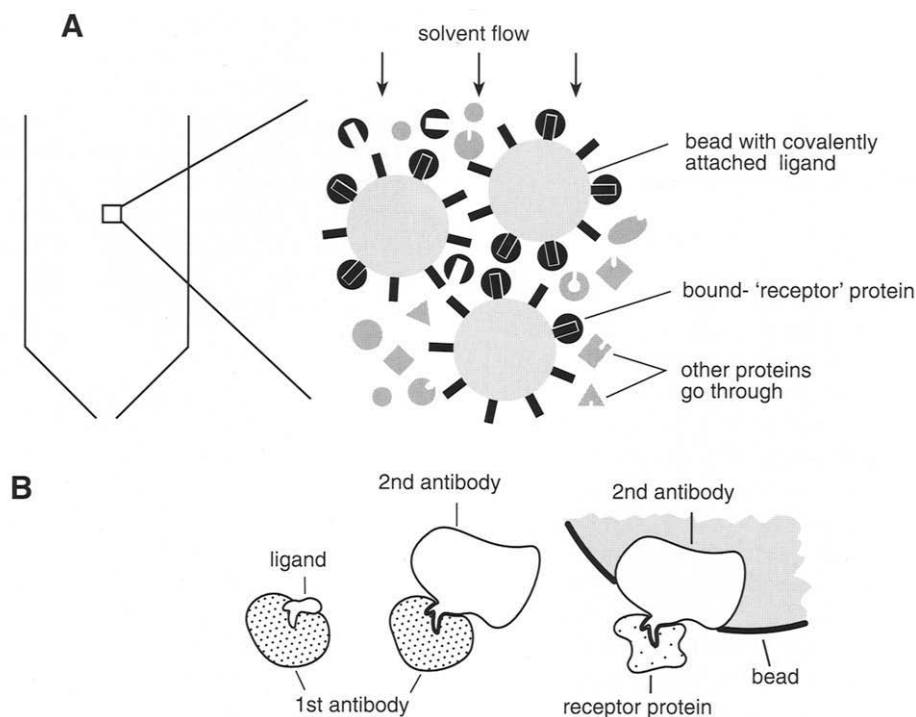


FIGURE A4-5 Affinity and immunoaffinity procedures for protein purification. (A) In affinity chromatography, the ligand is bound covalently to a substrate (an affinity bead), such as Sepharose. On passing the crude extract through the column, the protein of interest gets bound to the ligand, while the other proteins go through. The bound protein is subsequently eluted with salt or a solution of a hormone of higher affinity. (B) Immunoaffinity purification requires the preparation of anti-idiotypic antibodies, i.e., antibodies against the ligand are prepared; subsequently, antibodies against the first antibody are obtained. The second antibodies are attached covalently to a bead and the column is used for purification.

For immunoaffinity purification, anti-idiotypic antibodies (i.e., antibodies against antibodies to the ligand) are prepared and attached to a bead. The anti-idiotypic antibodies in fact mimic the ligand (Fig. A4-5B).

In preparation of an affinity or immunoaffinity column, care is taken to conjugate the hormone to the affinity bead, or protein (for use as an antigen), *via* an inactive part of the hormone molecule (e.g., in case of GAs, the C-16 CH₂, not the C-6 COOH). Such derivations are not always possible and sometimes essential groups have been used for derivatization.

2.2. Photoaffinity Labeling

A hormone may be derivatized in such a way that it becomes photoreactive, i.e., when activated by UV light, it forms a highly reactive species, which covalently links to a neighboring molecule. Such a photo-reactive hormone would bind to the "receptor" protein in the dark reversibly, but when photoactivated by UV, binding to the receptor would become covalent, thus making isolation and purification of the protein relatively easy (Fig. A4-6). Before use, it must be established that the biological activity of the photoaffinity-labeled hormone is indistinguishable from that of the unlabeled hormone in the dark. One cannot do any kinetic work on hormone binding using such derivatized hormones, but the binding protein can be isolated, and antibodies against it can be prepared. It can also be sequenced and its gene cloned. Photolytically active azido derivatives of auxins [e.g., 5-azido-3-indoleacetic acid (5N₃ IAA), where an N₃ group is substituted at C-5 of the indole ring], including tritiated derivatives, have been prepared and used for

isolating auxin-binding proteins. Despite the fact that photoaffinity labeling is a powerful technique for isolating proteins of interest (amid a mass of other proteins), it is not foolproof. Caution must be exercised and other criteria must be used before a protein so isolated can be considered to be specific for a hormone.

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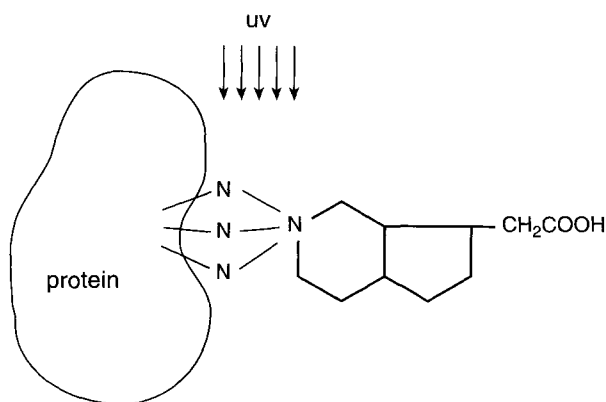


FIGURE A4-6 Photoaffinity labeling using 5-azido-3-indoleacetic acid. The protein fraction or cell extract is incubated with the azido derivative in the dark. After an appropriate incubation period, it is exposed to UV, which causes covalent links to be formed between the azido hormone and the protein of interest.

V**ENVIRONMENTAL REGULATION
OF PLANT GROWTH**

Plants are stationary organisms and have evolved to adjust their life strategies to various types of environmental signals. Among these signals, the perception of visible forms of radiation (light) and gravity and their effects on plant life are better studied. Our information on perception of other types of signals, UV radiation, temperature, wind, and the composition of atmospheric gases, is still very meager, although they all affect plant life.

Light is used by plants not only as a source of energy for photosynthesis, but also as a signal for growth and morphogenesis. Indeed, there is hardly any process in plant life, from seed germination to flowering, that is not affected by light. Light is also a very versatile signal, which varies not only in quality, but also in quantity, duration, and direction. Not unexpectedly, therefore, plants have evolved a variety of receptors that perceive the light signal in the red and/or blue/UV-A regions of the spectrum. The signals are transduced differently depending on the molecular structure of the photoreceptor. Types of photoreceptors, their roles in regulating plant responses, signal transduction pathways, and the interaction among photoreceptors and hormones are covered in Chapter 26. A newly emerging field is interaction among different photoreceptors, a situation analogous to that of interactions among hormones.

It is a common observation that plant shoots grow (or bend) toward a light source, whereas roots grow away from it; also that roots grow toward earth's gravity, whereas shoots grow away from it. These growth movements, phototropism and gravitropism, which occur in response to a directional stimulus (light or gravity, respectively), differ from other growth movements, which lack a directional component. They also differ from movements that occur in mature tissues and do not involve growth. Phototropic and

gravitropic responses differ from each other in their inducing stimuli and signal transduction pathways, but both lead eventually to uneven growth on two sides of a stem or root, a response that is mediated in part by the hormone auxin. These responses are covered in Chapter 27. Chapter 27 also provides additional information on photoreceptors in ferns, mosses, and algae and on some of the other types of growth and nongrowth movements.

Photoperception and Signaling

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1. LIGHT REGULATES MANY ASPECTS OF PLANT GROWTH AND DEVELOPMENT

Light affects nearly all aspects of plant life and development, from seed germination to flowering. The most well-known phenomenon, *photosynthesis*, involves

absorption of light in the visible spectrum by chlorophylls and accessory pigments and utilization of that energy to split water and generate ATP and NADPH for the reduction of carbon from CO_2 .

Other phenomena include growth or nongrowth movements in response to directional or diffuse light (see Chapter 27), measurement of the duration of light or darkness in a 24-h cycle (photoperiodism), and a diversity of responses including seed germination, seedling development (Fig. 26-1), and perception of neighboring vegetation, which are broadly included under the term photomorphogenesis. All these phenomena differ from photosynthesis in one very important respect: light is used as a signal for responses, not as an energy source to fix carbon. The energy requirement for most of these signals is not sufficiently high; in fact, in some cases, it is vanishingly small.

2. PLANTS HAVE EVOLVED AN ARRAY OF PHOTORECEPTORS

The light signal is the most versatile of all environmental signals. It can vary in four parameters: quality (wavelength), quantity (fluence or photons m^{-2}), directionality (unidirectional or diffuse), or duration (photoperiod or day length). These multiple facets of the light signal are overlaid by periodic phenomena, which emanate from the earth's rotation around its axis (diurnal periodicity) and around the sun (annual or seasonal periodicity). Plants perceive these varying signals by a variety of photoreceptors and transduce and integrate that information to modify their growth and development and to time their reproductive strategies.

Three types of photoreceptors are recognized: (i) phytochromes that absorb maximally in red (660 nm) and far-red (730 nm), although they also absorb in UV-A/blue; (ii) B/UV-A photoreceptors, which absorb principally in blue and UV-A parts of the spectrum (320–480 nm); and (iii) UV-B photoreceptors, which absorb in 280–320 nm. Among these, the phytochromes and two classes of B/UV-A receptors are better



FIGURE 26-1 The effect of white light on the pattern of development of bean seedlings. The seedling on the left was grown for 6 days in total darkness. It shows an etiolated phenotype, i.e., the seedling is pale yellow, leaves show a limited development, the hypocotyl is slender and tall, and the shoot apex is folded in a hook. The seedling on the right was grown for 6 days in white light. It shows a de-etiolated phenotype. The leaves/cotyledons are unfolded and expanded, green and photosynthetic, and the hypocotyl is sturdy and much shorter. Reprinted with permission from Smith (1976).

characterized. Very little is known about UV-B photoreceptors. This chapter deals principally with phytochromes and cryptochromes, one class of B/UV-A receptors. Chapter 27 deals with other classes of B/UV-A photoreceptors. Photoperiodic phenomena, including flowering, are not covered in this book.

Fluence of light and action spectra of light-induced responses are important concepts for an understanding of photoreceptors. These concepts are explained in Box 26-1.

BOX 26-1 FLUENCE AND ACTION SPECTRA

THE FLUENCE RATE (OR photon flux density) can be expressed in terms of radiant energy as watts per square meter (W m^{-2}) [note: the unit time (seconds) is contained within the term watt: $1\text{W} = 1 \text{ joule s}^{-1}$] or in terms of photons (quanta) as moles $\text{m}^{-2} \text{s}^{-1}$. Fluence (moles of photons m^{-2}) is a function of fluence rate \times irradiation time. While photochemical reactions are usually expressed in energy units, biological responses are often expressed in terms of photons (quanta in the visible range).

Biological responses vary in their fluence requirements. Some biological responses are initiated and saturated at very low fluences of light, as little as 0.1 nmol m^{-2} of red light, equivalent to a few seconds of moonlight. These are referred to as *very low fluence responses* (VLFRs). The majority of photomorphogenic responses, however, are not initiated until fluences reach about $1.0 \mu\text{mol m}^{-2}$ and typically saturate at fluences between 10.0 and $100.00 \mu\text{mol m}^{-2}$. These are still relatively small amounts of energy, which can be supplied in one or more brief flashes of light. They are referred to as *low fluence responses* (LFRs). The third class of responses, known as *high irradiance responses* (HIRs), require much higher fluences of light to saturate, typically about 100-fold higher fluences than LFRs ($\sim 10 \text{ mmol m}^{-2}$ or more). The fluence rate is not necessarily high, in fact it may be lower than that for LFRs, but they require continuous exposure for a long time—hours, not minutes (Fig. 26-2).

VLF and LF responses differ from HI responses in another important respect. One of the laws of photochemistry, the law of reciprocity, states that duration of exposure and fluence rate show reciprocity in the level of effectiveness; i.e., if the arithmetical product of fluence rate (in W m^{-2}) \times duration is held constant, then a brief irradiation at a high fluence rate produces the same level of effect as longer irradiation at a low fluence rate. The response is independent of temperature, although its display may well be affected by it. If such a relationship is found in a response, it indicates that only one type of photoreceptor is involved. For biological responses, the law can be restated—the magnitude of a response is the same for any combination of exposure time and fluence rate as long as the fluence is the same. Biological responses are much more complicated than simple photochemical reactions; nonetheless, by measuring the level of response under different combinations of exposure times and fluence rates, while keeping the total fluence the same, it can be shown that VLFRs and LFRs show reciprocity, whereas HIRs do not (Table 26-1). For HIRs, the response bears no direct relationship to fluence and may continue to increase with exposure time or irradiance.

In nature, although some responses are mediated by VLF or LF and require relatively short exposure times, most responses progress under continuous illumination and, thus, are HIRs.

Responses vary in their action spectra. An action spectrum indicates which wavelengths of irradiation are most effective in eliciting a response. An action spectrum is constructed because it allows a comparison with absorption spectra of known pigments and thus an insight into the nature of the photoreceptor involved. Since any simple photoresponse is a function of fluence rate and duration of irradiation, the most effective wavelengths are those that require the fewest number of photons to elicit a certain level of response. If the “response” is plotted against a wavelength, as the reciprocal of the number of incident photons required to produce a given effect, then peaks in the resulting spectrum will represent the most effective wavelengths and allow direct comparison with peaks in absorption spectra.

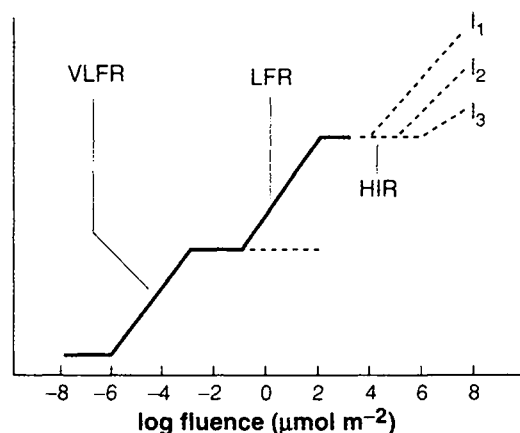


FIGURE 26-2 Three types of photomorphogenic responses. The relative magnitude of responses is plotted against increasing fluences of monochromatic red light. Short light pulses activate VLF and LF responses. Since HIRs are also proportional to irradiance, the effects of three different irradiances given continuously are illustrated ($I_1 > I_2 > I_3$). From Briggs *et al.* (1984).

TABLE 26-1 Types of Light Responses and Reciprocity

Type of response	Range ($\text{mol} \cdot \text{m}^{-2}$)	Reciprocity
Very low fluence	0.1 nmol–1.0 μmol	Yes
Low fluence	1.0–100 μmol	Yes
High irradiance	>10 mmol	No

The first step in the determination of an action spectrum is the construction of fluence rate–response curves for different wavelengths (usually a 10-nm wave band) of irradiation (Fig. 26-3). From these curves, photon fluences required to produce a defined magnitude of response, a “standard” response, is determined for each wave band (i.e., the number of photons required to produce say 50% germination, or 50% inhibition of growth). The action spectrum is then prepared by plotting the reciprocals of these photon fluences against the wavelength.

While action spectroscopy has been used to great effect in demonstrating the involvement of certain pigments in specific processes (e.g., carotenoids in vision and DNA repair, and chlorophyll in photosynthesis), its application to photomorphogenesis has met with limited success for two reasons: (i) The photoreceptors are present in much lower amounts than say chlorophylls and (ii) the absorption spectrum of a chromophore or pigment is affected greatly by its molecular environment—*in vitro* by the type of solvent and *in vivo* by its association with other molecules. Therefore, it is usually impossible to obtain an exact match between *in vitro* absorption and *in vivo* action. As we will see for phytochrome, action spectra did concentrate attention on the appropriate regions of the spectrum, but it was the light-reversible changes in absorption properties that led to the identification of the photoreceptor. Blue light receptors have been more difficult to identify and, those that have been, were identified using molecular genetic techniques.

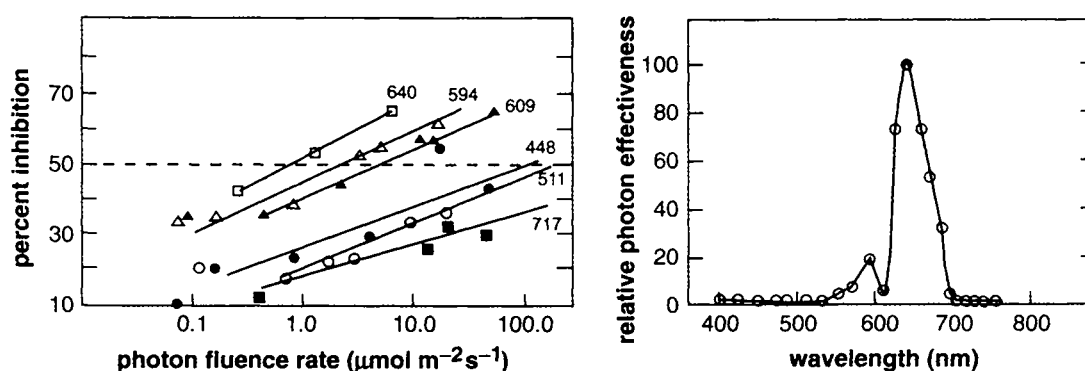


FIGURE 26-3 Representative fluence rate–response curves and an action spectrum (based on such curves) for light inhibition of hypocotyl growth. A relatively simple spectrum with inhibition only by red light is shown. From Beggs *et al.* (1980).

3. DISCOVERY OF PHYTOCHROME

In the 1930s, Flint and McAlister at the U.S.D.A. Seed Testing Laboratory observed that seed germination in a certain variety of lettuce was promoted by irradiation with red light and inhibited by far-red light. These observations were taken up by H.A.

Borthwick, a botanist, and S.B. Hendricks, a physical chemist, at the U.S.D.A. Research Station at Beltsville, Maryland, whose collaboration in the 1940s and 1950s led to one of the major discoveries of all time. In a series of elegant experiments, these scientists established that red/far-red reversibility was a characteristic of many morphogenic responses and that phytochrome was the photoreceptor involved.

The group led by Borthwick and Hendricks constructed action spectra for several different responses, including induction of flowering in short- and long-day plants, inhibition of elongation in stems, and seed germination in lettuce (Fig. 26-4). The spectra showed clearly that the action maxima for promotion or inhibition occurred, very nearly, at the same wavelengths in all responses—about 660 nm in the red and 730 nm in the far-red region of the spectrum (the region between 700 and 800 nm is referred to as the far-red region). Furthermore, for each response, light of the two wavelengths gave opposite results. These data established that a low energy photoresponse, with opposite actions in the red and far-red regions of the spectrum, occurred widely in flowering plants.

Then, in 1952, came perhaps the most momentous discovery in the history of photomorphogenesis: the responses to the two wavelengths were not only

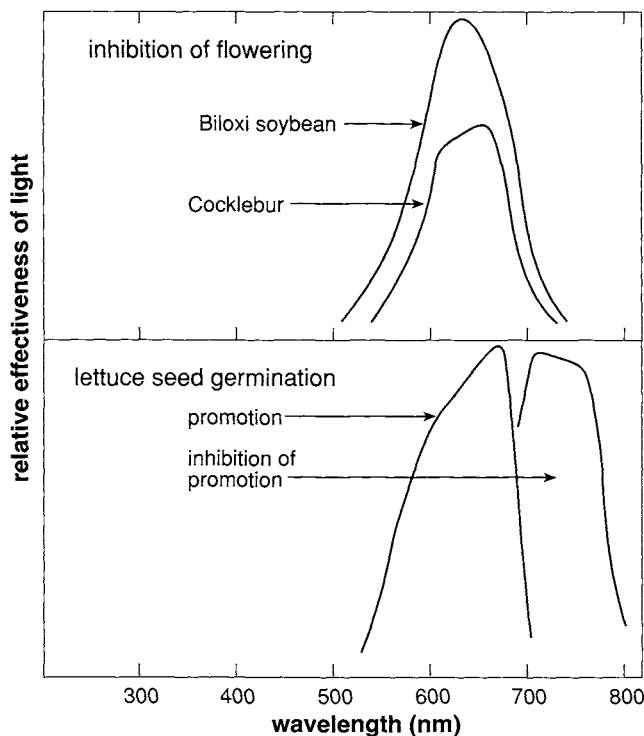


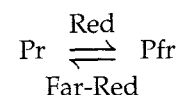
FIGURE 26-4 Action spectra for flowering and seed germination. (Top) Action spectra for suppression of floral induction in Biloxi soybean and cocklebur, two short-day plants. These plants require a minimum of 14 or 12 h of uninterrupted darkness for induction of flowering, respectively. An interruption of the dark period by light of any wavelength of sufficient energy would inhibit flowering, but the most efficient wavelengths, i.e., those requiring the least number of photons, are those in the 640 to 660-nm region. (Bottom) Promotion and inhibition of germination in Grand Rapids lettuce seeds. The most efficient wavelength for promotion and inhibition is 660 nm and between 710 and 750 nm, respectively. Adapted from Sage (1992).

opposite to each other, they were also antagonistic. In a classic experiment, Borthwick and Hendricks found that lettuce seeds given a pulse of red light and hence potentiated to germinate would not germinate if the red light treatment was followed quickly with a pulse of far-red light. Furthermore, this reversal could be continued repeatedly, the seeds would germinate if the last light treatment was red light and would not germinate if the last light treatment was far-red light (Fig. 26-5). It was as if the seeds remembered the last light that they had received.

Since the light fluences required for these responses were low, about $1.0 \mu\text{mole m}^{-2}$, far too low for photosynthesis, it was obvious that a new pigment, not chlorophyll, was involved; also, chlorophyll was absent in etiolated seedlings. The similarity of action spectra for seed germination, inhibition of stem elongation, and flowering indicated that the same pigment system was involved in all three responses.

The question of whether there were two different photoreceptors, one absorbing maximally at 660 nm and the other at 730 nm, or whether there was only one photoreceptor with two interconvertible forms could not be answered definitively until the development of a dual wavelength spectrophotometer. This instrument could treat a sample with red or far-red radiation (these are called the “actinic beams”) and then measure the absorbance of the sample at red or far-red wavelengths. The protocol was to irradiate the sample with red light, determine the visible absorption spectrum, repeat the procedure using far-red light, and calculate what is known as a difference spectrum (Fig. 26-6).

The results were clear. Red light caused a decrease in the absorption maximum in the 660-nm region and an increase in the 730-nm region, whereas far-red light reversed these spectral changes. One photoreceptor cycled between two absorbing forms: one absorbing principally in the red and the other in the far-red region, as shown below. Confirmation was obtained by showing that buffered aqueous extracts of etiolated tissue contained a substance that gave similar spectral changes on irradiation with red and far-red light. This substance was partially purified, shown to be a chromoprotein, and christened phytochrome (Gk for “plant color,” a modest name for a pigment that turned out to be of extreme importance to plant life). The red-absorbing form was called Pr, and the far-red absorbing form was called Pfr.



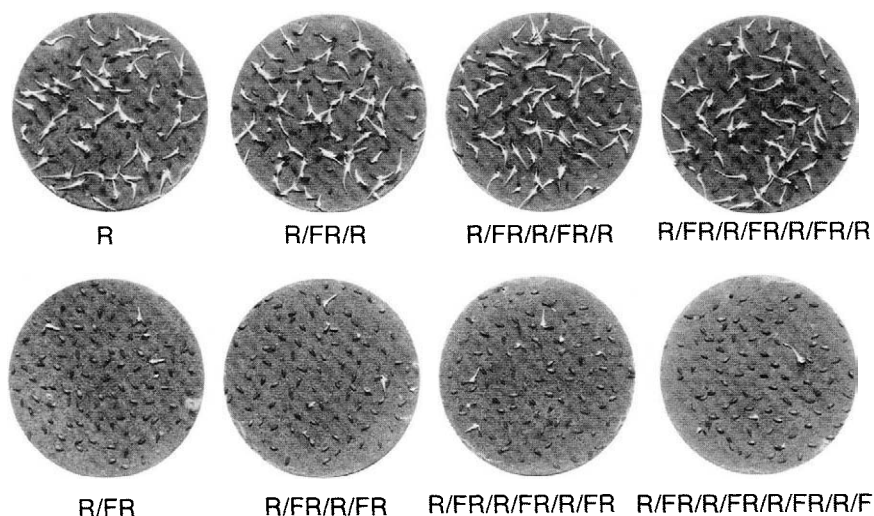


FIGURE 26-5 Red/far-red reversal of lettuce (*Lactuca sativa* cv Grand Rapids) seed germination. Most seeds germinate if the last irradiation is by red (R) light; germination is minimal if the last irradiation is by far-red (FR) light From Sage (1992).

R/FR reversibility is seen in many, although not all, responses mediated by phytochromes. It is referred to

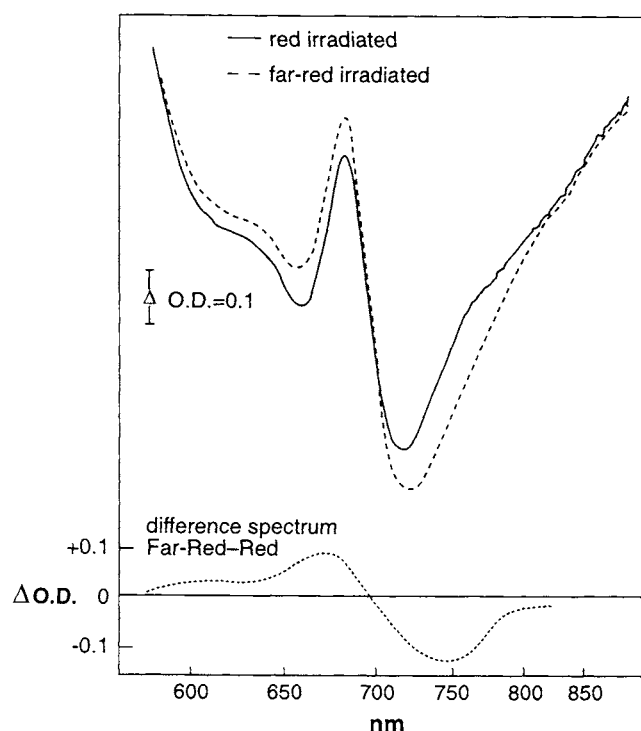


FIGURE 26-6 Absorbance changes after sequential red and far-red light treatments of etiolated maize coleoptiles. Upper curves show absorption spectra, whereas the lower curve shows the difference spectrum, calculated by subtracting the "red" absorption spectrum from the "far-red" absorption spectrum. The difference spectrum shows that red light increases the absorbance of the tissue at 730 nm while decreasing it at 660 nm. From Sage (1992).

as the "phytochrome signature." If an uncharacterized response shows the phytochrome signature, that is the first indication that it may be phytochrome mediated. The reverse, the absence of the signature, does not mean, however, that the response may not be phytochrome mediated.

The discovery of phytochrome in 1959 quickly led to demonstration of its presence in other flowering plants, as well as in gymnosperms, ferns, mosses, and algae (phytochrome seems to be absent in fungi). At the same time, the number of responses mediated by phytochrome quickly multiplied. Two approaches were used for these demonstrations: the criterion for red/far-red reversibility and, as extraction methods became more refined, monoclonal antibodies against specific epitopes of phytochrome could be prepared and used immunologically.

SECTION I. PHYTOCHROMES AND CRYPTOCHROMES

1. PHYTOCHROME FAMILY IN ANGIOSPERMS

Early experiments on phytochrome were done *in vivo* using etiolated materials (e.g., oat or corn coleoptiles, turnip, or pea stems)—etiolated because light-grown tissues have many other pigments, chlorophylls, carotenoids, and anthocyanins, which also absorb in red and blue regions of the spectrum and thus interfere with the measurement of phytochrome. Accordingly, the phyto-

chrome that was isolated and purified, and whose gene was first cloned, was the phytochrome that was abundant in etiolated plants. This phytochrome is now known as phytochrome A or Phy A (or type I phytochrome); it is the only member of type I phytochrome family identified so far. Even before the cloning of the *PHYA* gene, there were reasons to believe that other phytochromes occurred in green or light-grown tissue. For some time, these phytochromes were referred to as "green" or type II phytochromes. Cloning of the *PHYA* gene from oat and rice, and probing of the *Arabidopsis* genomic DNA library with conserved sequences from oat cDNA, led to a confirmation that we are not dealing with just one phytochrome, but with distinct members of a small, but divergent family. In *Arabidopsis*, the apoprotein moiety of the phytochrome is coded for by five distinct genes—*PHYA*, *PHYB*, *PHYC*, *PHYD*, and *PHYE*—which occur in single copies (for the system of naming phytochrome genes, apoproteins, mutants, etc., see Box 26-2). Homologous genes, and many novel ones, are now known from other dicots and monocots and from conifers, ferns, mosses, and algae.

1.1. Structure and Synthesis of Phytochromes

Phytochrome molecules are soluble chromoproteins that are organized along a similar structural pattern in

all plants investigated. This section describes the structure, synthesis, and assembly of phyA because more is known about phyA than any others; differences that are known are indicated at appropriate places.

The phytochrome molecule has two components: a protein part (the apoprotein) and a chromophore. The molecular mass of the phyA apoprotein in different species is about 125 kDa (range from 118 to 130). The apoprotein is folded into two structural domains: a slightly larger N-terminal domain, which carries the chromophore, and a smaller C-terminal domain (Fig. 26-7A). The two domains are linked to each other *via* a short hinge-like segment, which is susceptible to proteolysis during extraction. Carefully extracted phytochrome has a molecular mass of ~250 kDa, which means that the native molecule occurs as a dimer in solution. Small-angle X-ray scattering has provided a model of a phyA dimer with two identical units. Each monomeric unit has a larger N-terminal domain which appears as an oblate ellipsoid, while the smaller C-terminal domain appears as a flat disc. The two domains in a monomer are connected to each other at their edges (the hinge region), while the two monomers are attached to each other via their carboxy termini. Transmission electron micrographs of phyA show the molecule as clusters of two, three, or four spots, depending on the angle at which the dimer is viewed (Figs. 26-7C and 26-7D).

BOX 26-2 A SYSTEM OF NOMENCLATURE FOR PHOTORECEPTORS

THE EXISTENCE OF SEVERAL phytochromes, their holoproteins, apoproteins, genes, mutants, occurrence of phytochromes in their red-or far red-activated forms has led to a system of nomenclature. This system adopted by most authors on phytochromes is given below [for details, see Quail *et al.* (1994)].

- i. Holoproteins are written in small case letters, but the type of phytochrome is designated by the following capital letter, e.g., phyA, phyB, and phyC.
- ii. Wild-type genes for apoproteins are written in capital letters and italicized, e.g., *PHYA*, *PHYB*. If more than one gene of the same class occurs in a plant, the genes are numbered, e.g., *PHYB1* and *PHYB2*.
- iii. The encoded apoproteins are written in capital letters, but are unitalicized, e.g., PHYA and PHYB.
- iv. Mutations in specific genes are designated by small case letters and are italicized, e.g., *phyA* and *phyB*; mutant alleles are numbered, e.g., *phyA-1* and *phyB1-1*.
- v. The Pr and Pfr forms of phyA are denoted as PrA and PfrA, respectively; those of phyB as PrB and PfrB, respectively; and so on.

In this text, the above system is used for phytochromes. The same system is also used for cryptochromes and other blue/UV-A receptors, e.g., cry1 and cry2 for holoproteins of cryptochrome 1 and cryptochrome 2, respectively; *CRY1* and *CRY2* for their genes, CRY1 and CRY2 for the apoproteins, etc. The reader is cautioned that some authors still use *PHYA*, *PHYB*, *CRY1*, or *CRY2* for holoproteins.

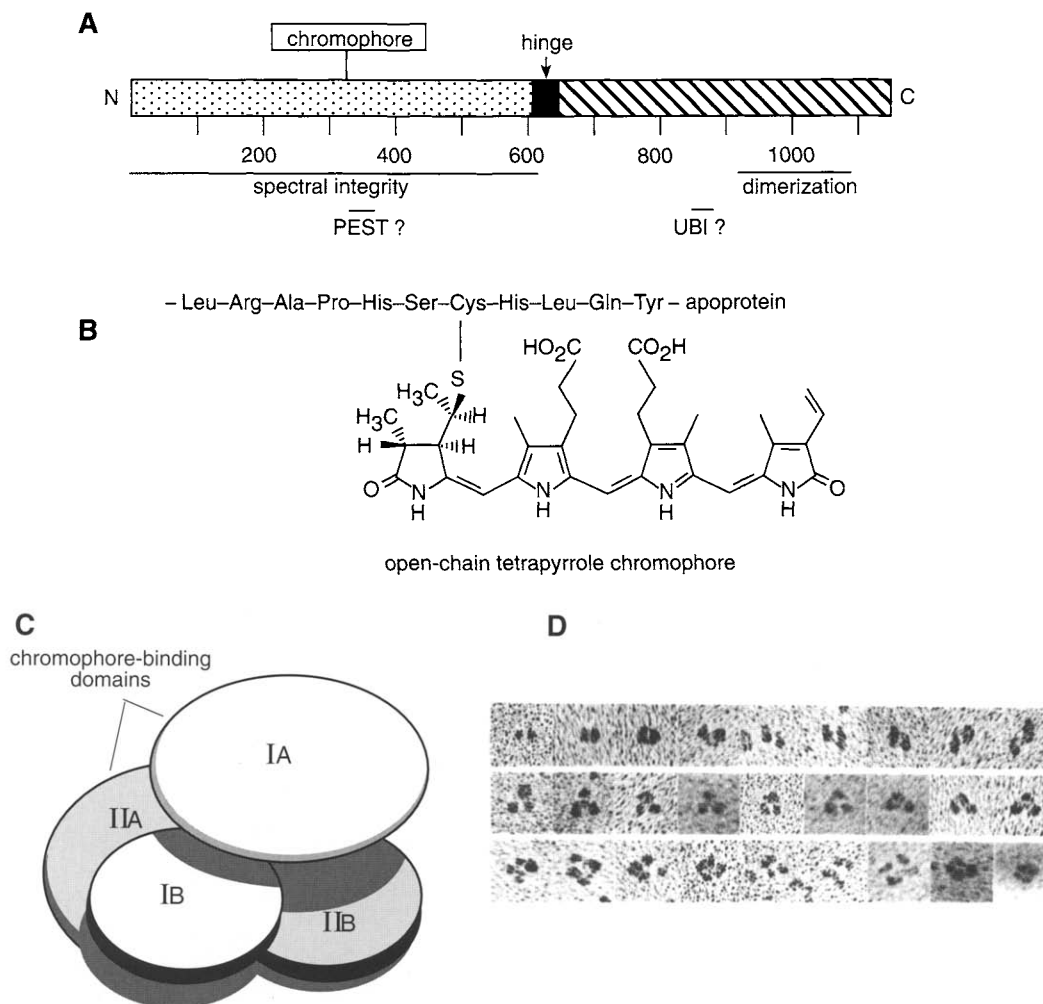


FIGURE 26-7 Schematic illustration of the structure of phytochrome A. (A) The apoprotein is divided into an N-terminal and a C-terminal half separated by a hinge region. The N-terminal carries the chromophore and is important for spectral integrity. The C-terminal is important for dimerization and for signaling. The presumed location of the PEST sequence and the site for ubiquitin conjugation are indicated. (B) The chromophore, phytylphytylphytylphytyl (PΦB), is a linear tetrapyrrole, which is attached via a thioester linkage to a conserved cysteine in the apoprotein. (C) A molecular model of pea phytochrome in the red light absorbing form based on small-angle X-ray scattering. This mode of viewing is suitable for large molecules that cannot be crystallized. The model is shown as a dimer with two identical monomers labeled I and II. Each monomer has a larger chromophore-bearing N-terminal domain (IA and IIA) shaped like an oblate ellipsoid, and a smaller C-terminal domain (IB and IIB) shaped more like a disc. The two domains in a monomer are attached to each other at their edges, while the lower flat plane of disc IB is in contact with the upper flat plane of IIB. (D) Transmission electron micrographs of extracted and purified phyA molecules from pea. The purified preparations were spread on mica wafers, platinum shadowed, and carbon replicas made for observation under the microscope. The molecules attach to the mica flakes in various manners and appear as dimeric (top row), trimeric (middle row), or tetrameric (bottom row) clusters, depending on the particular angle at which the model in (C) is viewed. A and B and from Chory (1997) and C and D and from Tokutami *et al.* (1989) and courtesy of Masamitsu Wada, Tokyo Metropolitan University, Tokyo.

Sequence comparisons among phytochrome apoproteins indicate that the N-terminal domain with the chromophore is highly conserved among different *PHY* gene families, whereas the C-terminal domain is variable. Site-directed mutagenesis and/or expression of deletion constructs in transgenic plants, i.e., the

coding sequence is deleted in part and the remainder is expressed as a transgene, has further revealed that the amino acids flanking the chromophore attachment site are able and sufficient to autocatalyze covalent ligation of the chromophore to the apoprotein. Moreover, the N-terminal domain of ~ 600 amino acids,

including the chromophore, is involved in photoperception, as well as R/FR reversal (spectral integrity), whereas the C-terminal domain is involved in dimerization of the monomers and in signal transduction (see Fig. 26-7A). Other domains important for biological activity and signaling are indicated later in Section III.

The chromophore is an open chain tetrapyrrole, known as phytochromobilin (PΦB), and is attached to the apoprotein at a conserved cysteine residue (Fig. 26-7B). The PΦB chromophore is similar in structure to phycocyanobilin (PCB), which is the chromophore in blue-green algae. Both differ from the Mg^{2+} -dependent ring tetrapyrrole, which constitutes the chromophore part of the chlorophyll molecule.

Whereas the apoprotein is encoded by its gene (e.g., *PHYA*, *PHYB*) and synthesized in the cytoplasm from its mRNA, the chromophore is synthesized in the plastid (Fig. 26-8). On export into the cytoplasm, the chromophore is ligated covalently to the apoprotein. Since the apoprotein contains within it amino acid sequences that are able to autocatalyze this covalent attachment, it is possible to express a cDNA encoding the apoprotein in transgenic plants, yeast, *Escherichia coli*, etc., and, on an exogenous supply of chromophore precursors, obtain a spectrally functional phytochrome. This technique has proven very useful in the dissection of phytochrome signaling.

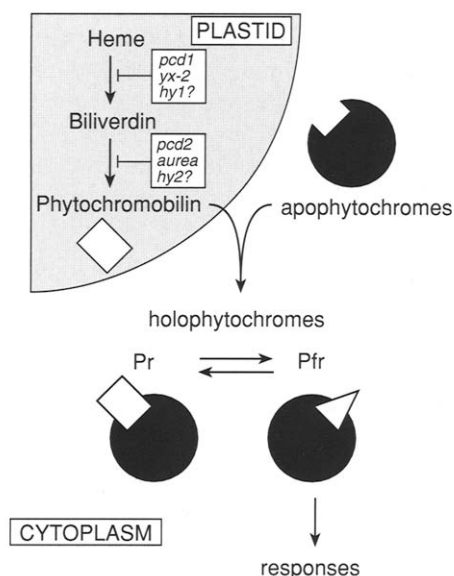


FIGURE 26-8 Synthesis of phytochromobilin in the plastid and assembly of the phytochrome holoprotein in the cytoplasm. The chromophore is synthesized from 5'-aminolevulinic acid with heme and biliverdin as intermediates. Some mutants defective in the synthesis of phytochromobilin are shown in square boxes inside the plastid. Reprinted with permission from Terry (1997), © Blackwell Science Ltd.

1.2. Absorption Spectra and Pfr/P Ratios

Phytochrome holoproteins have major absorption peaks in red or far-red regions, although they also have minor peaks in the blue and ultraviolet regions of the spectrum (Fig. 26-9). The Pr form absorbs maximally in the red region (660–667 nm) and a little also in the far-red region (although not above 710 nm). The Pfr form has maximal absorbance in the far-red region (~730 nm) and also has significant absorption in the red region. The 730-nm absorbance for Pfr is unique. Both forms also absorb in B/UV-A (~370–400 nm) and UV-B (~270 nm) parts of the spectrum, but the biological activity of phytochromes is believed to be due mostly to their red/far-red absorbance.

Since both forms absorb in the red region and the Pr form also absorbs a little in the far-red region, neither of the phototransformations can be driven to completion and, under saturating irradiation, both forms are present in a dynamic equilibrium known as the *photostationary state*, denoted by the symbol Φ . The symbol Φ refers to the ratio of Pfr to total phytochrome (Pfr/P) in a light environment. The Pfr to total phytochrome ratio, under saturating R, varies among species and under different conditions, but is usually about 80%, or $Pfr/P = 0.8$. In contrast, because Pr has only a limited absorption in the far-red region, the $Pfr \rightarrow Pr$ conversion under saturating doses of FR light can be as high as 97%, or $Pfr/P = 0.03$. The transformation of Pr to Pfr is not a one-step process, but occurs via a series of short-lived intermediates. Similarly, transformation of the Pfr form to the Pr form also occurs via a series of intermediates, which are not the

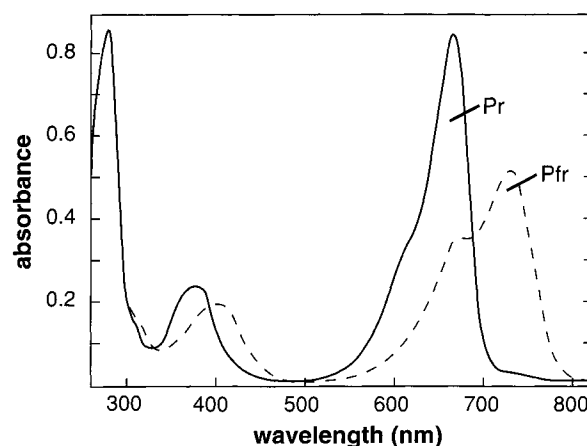


FIGURE 26-9 Absorption spectra of Pr and Pfr forms of phytochrome *in vitro*. The Pr form absorbs maximally in the red, whereas the Pfr form has maximal absorbance in far red (~730 nm). Both forms also absorb in B/UV-A and UV-B parts of the spectrum. Neither form absorbs significantly in green; hence, green safe lights at low fluence are often used for work on phytochrome. From Chory (1997).

same as in the Pr to Pfr transition. In continuous white or blue or red/far-red light, the Pr and Pfr forms cycle back and forth, and these intermediates can accumulate to significant amounts. For these reasons, the value of Φ in sunlight is closer to 0.6.

The photochemistry of the Pr to Pfr transformation has been intensively investigated using a variety of spectroscopic techniques. The consensus of these studies is that on absorbance of red light, the geometry of the methine bridge between the C and D rings of the P Φ B changes from C₁₅ = C₁₆Z in Pr to C₁₅ = C₁₆E in Pfr (Fig. 26-10). Studies using synthetic analogs of the chromophore indicate that each pyrrole ring of the linear tetrapyrrole plays a different role. For instance, ring A anchors the chromophore to the apoprotein, ring B has a role in the positioning of the chromophore within a cleft in the apoprotein, and ring D plays a role in the photoreversible spectral change. The *cis-trans* isomerization in the tetrapyrrole brings about changes in the apoprotein α -helical structure which start a signaling cascade. Other studies also indicate that the apoprotein moiety undergoes conformational changes on Pr to Pfr transformation. For example, certain domains of the apoprotein become more exposed to proteolysis or to phosphorylation by exogenous protein kinases. The Pr and Pfr forms also differ immunologically.

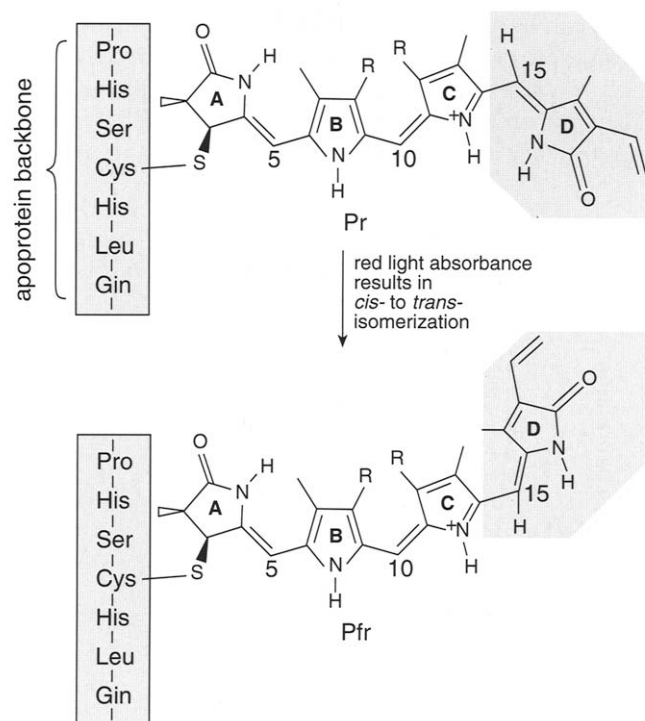


FIGURE 26-10 Conformational changes in chromophore. The chromophore undergoes a *cis-trans* isomerization at carbon 15 in response to R and FR light. Carbons 5, 10, and 15 are numbered. Adapted with permission from Andel *et al.* (1996).

1.3. Light Stability and Selective Degradation of Phytochrome A

Among all the phytochromes, phyA is singularly unstable in light. It is synthesized in the dark in the Pr form (PrA) and is relatively stable in the dark with a half-life ($t_{1/2}$) of ~ 100 h. However, on conversion to the Pfr form (PfrA) in red or white light, it is much less stable and is degraded rapidly with a $t_{1/2}$ of ~ 60 min or 1/100 that of Pr. This drop is due to a combination of three factors. First, phyA in the Pfr form downregulates the transcription of its own gene (Fig. 26-11). Second, the *PHYA* mRNA is degraded in the light environment. Third, certain conformational changes occur in PfrA that predispose it to selective destruction by the proteolytic machinery in the cell.

The destruction of phyA occurs *via* polyubiquitination of PHYA apoprotein and its hydrolysis by the 26S proteasome machinery in the cell (see Box 26-2 and Fig. 22-14 in Chapter 22). A specific region in the C terminus half of phyA, which is rich in lysine residues, is thought to be involved in conjugation to ubiquitin (UBI site in Fig. 26-7A). This region is exposed in the conformational change that occurs in phyA upon conversion to Pfr; it is conserved among all PHYA apoproteins, but is not present in apoproteins of the light-stable phytochromes, such as phyB or phyC.

Among the first signs of PfrA degradation is a sequestration of phyA molecules into relatively large particles ($\sim 1 \mu\text{m}$), large enough to be seen under a light microscope. Using antibodies against phyA epitopes, such sequestration can be seen within minutes of an exposure of etiolated material to red or white light. The reasons for sequestration are but unknown, it is possible that it results from ubiquitination of PfrA. It has been shown that phyA molecules are ubiquitinated rapidly upon exposure to red or white light. It has also been shown that while an intact C-terminal domain is essential for phyA degradation, specificity for degradation is provided by the N-terminal domain.

Another motif common to proteins with short half-lives is the PEST sequence (named after amino acids Pro, Glu, Ser, and Thr). The PEST sequence is also highly conserved among phyA molecules, but is absent from the apoprotein sequences of phyB and phyC. The PEST sequence in PHYA is located close to the chromophore (see Fig. 26-7A) and is believed to be exposed in the PfrA.

All phytochromes other than phyA, i.e., phyB–phyE in *Arabidopsis* and their homologues or similar types in other plants studied so far, are light-stable, type II phytochromes. Light-stable phytochromes usually occur in much lower abundance in etiolated materials than phyA, but as a result of phyA degradation in light, the

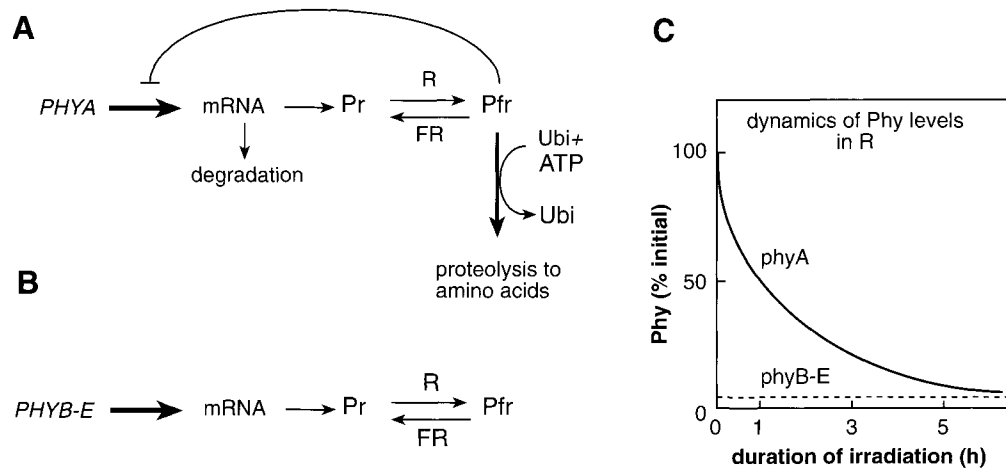


FIGURE 26-11 Light-dependent regulation of phytochrome expression. (A) Synthesis and regulation of light-labile phyA. PrA accumulates in dark-grown seedlings due to high levels of *PHYA* transcription and the long half-life of PrA. PhyA levels decrease in light due to the feedback repression of *PHYA* transcription, constitutive degradation of *PHYA* mRNA, and selective degradation of PfrA by the ubiquitin/26S proteasome pathway. (B) Synthesis of light-stable phyB-E. (C) Typical effect of continuous R irradiation on phyA and phyB-E levels in etiolated seedlings. Reprinted with permission from Clough and Vierstra (1997), © Blackwell Science Ltd.

relative proportions change in light. Some calculations indicate that the proportions of phyA:phyB change from ~ 10:1 in dark to about 1:1 in light. Despite substantial reductions in its levels, however, phyA still remains one of the major phytochromes in light.

Analysis of the promoter of *PHYA* gene provides an explanation. The promoter has three transcription initiation sites which result in production of transcripts of three sizes. The shortest and most abundant transcripts are down regulated by light in the shoot region; moreover, this light regulation requires the presence of both phyA and phyB. The other two minor transcripts are expressed in the shoot and all three are expressed in the root, but the expression of these transcripts occurs constitutively.

1.4. Tissue-Specific Expression of *PHY* Genes in Angiosperms

Immunocytochemical studies, using antibodies specific to PHYA apoprotein, indicate that the phytochrome is distributed throughout most plant tissues and that its concentrations are especially high in young, meristematic regions. Gene-specific probes in *Arabidopsis* and tomato show that, except for the *PHYA* gene, the expression of which is inhibited significantly in light, transcripts of other *PHY* genes show no significant change in their steady-state levels in light or dark and, thus, seem to be expressed constitutively (Fig. 26-12, left). *PHY* genes are expressed in most tissues (Fig. 26-12, right), although as more data accumulate, there is evidence for developmental and tissue

specificity in their expression. Thus, the mRNAs of *PHY* genes occur in low to negligible amounts in unimbibed tomato seeds, but their levels rise dramatically with imbibition.

1.5. Evolutionary Relationships among *PHY* Genes

Based on deduced amino acid sequence data, *PHY* genes in angiosperms are divided into four classes, represented by *PHYA*, *PHYB/D*, *PHYC*, and *PHYE* of *Arabidopsis*. The number of classes may turn out to be more in the future, but the number of genes in each class is known to vary widely among angiosperms. For example, in tomato, five genes, *PHYA*, *PHYB1*, *PHYB2*, *PHYE*, and *PHYF*, have been cloned and many others are suspected, but *PHYB1* and *PHYB2* are not orthologous to the *PHYB/D* group in *Arabidopsis* and *PHYF* is new. In tobacco, two *PHYA* genes, *Nt-PHYA1a* and *Nt-PHYA1b*, and several *Nt-PHYB* genes are reported; in oat, three *PHYA* genes have been identified. In general, the dicots studied show a larger number of phytochrome genes than the monocots, and so far no representative *PHYE* genes have been isolated from the latter group.

There is not much sequence homology among members of different classes (~50–60% amino acid identity), with no significant stretches of conserved sequences. In contrast, encoded polypeptides of the same class gene in different plants show a much greater homology, e.g., *PHYB* from rice and *Arabidopsis* are more than 70% homologous. The hydrophathy

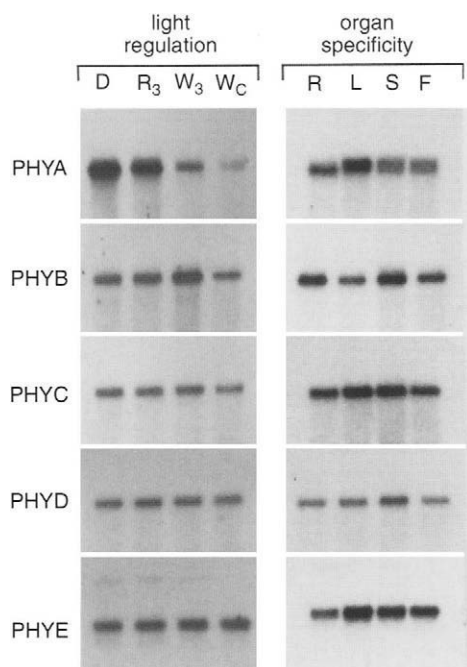


FIGURE 26-12 Northern blot analysis of various *PHY* mRNAs in *Arabidopsis*. (Left) Light regulation of *PHY* gene expression. RNA was isolated from 7-day-old seedlings grown in continuous darkness (D), in the dark but exposed to 5 min of red light 3 h before harvest (R_3), grown in the dark but exposed to 3 h of continuous white light before harvest (W_3), or grown under 7 days of continuous white light (W_c). Note that there is no significant change in the abundance of *PHYB*–*PHYE* RNA, but there is a noticeable decline in *PHYA* RNA in seedlings given 3 h of R or W light before harvest, or those grown in continuous white light for 7 days. (Right) Tissue-specific expression of *PHY* genes. RNA was isolated from roots (R), rosette leaves (L), flowering bolt stems (S), or whole flowers (F). Plants were grown for 2 weeks in liquid culture (for sampling of roots), or on soil for 2 weeks (for leaves) or 3–4 weeks (for stems and flowers). A 1- μ g portion of poly(A)-selected RNA was loaded per lane, and gene-specific probes were used for hybridization. From Clack *et al.* (1994) with kind permission from Kluwer.

profiles of different class apoproteins, however, are similar, which suggests that the three-dimensional structure of the proteins has been conserved during evolution.

Sequence alignments of deduced amino acids from full-length cDNA clones have enabled phylogenetic distance trees to be constructed for angiosperms, as well as more comprehensive ones that include conifers, ferns, mosses, and algae. For further details, the reader is referred to Mathewes and Sharrock (1997).

2. THE CRYPTOCHROME FAMILY

Many plant responses are mediated by blue light. Examples include phototropic curvature in response

to unidirectional light, de-etiolation response in etiolated seedlings, induction of chalcone synthase (*CHS*) and other flavonoid synthesis genes (e.g., *DFR* gene encoding dihydrofolate reductase), and promotion of stomatal opening. Not all these responses are mediated by the same blue UV-A receptor. At least three different classes of B/UV-A receptors are already known and they mediate different responses (Table 26-2). The following section deals with only one class of B/UV-A receptors, the so-called cryptochromes, because they share some of the responses with phytochromes. The other B/UV-A photoreceptors are covered in Chapter 27.

2.1. Cloning of *CRY1* and *CRY2* Genes

Several photomorphogenic mutants of *Arabidopsis* were isolated in a screen for an etiolated phenotype among seedlings grown in white light. These mutants, known as *hy* (for long hypocotyl), were shown later to be of several different kinds (see section II, 1). Further screening of *hy* mutants with different wavelengths of monochromatic light revealed that the *hy4* mutant was insensitive to hypocotyl growth inhibition by blue light, while still being susceptible to inhibition by R, FR, and W lights. T-DNA tagging of a *hy4* allele led to isolation of the wild-type *HY4* gene, which on further analysis was shown to encode the apoprotein for a blue light photoreceptor. The photoreceptor was named cryptochrome 1 or *cry1*, and the *HY4* gene was renamed *CRY1*. The cloning of *CRY1* led to isolation of *CRY2* from an *Arabidopsis* genomic library. *CRY1* and *CRY2* orthologs have also been cloned from tomato, and related sequences are known from mustard (*Sinapis alba*) and a green alga, *Chlamydomonas*.

2.2. Structure of Cryptochromes

Cry1 and *cry2* are structurally similar proteins, although *cry2* is smaller. Both proteins have an N-terminal domain, which is involved in light perception, and a C-terminal domain, which is involved in signaling (Fig. 26-13). N-terminal domains bear some similarity

TABLE 26-2 Known B/UV-A Photoreceptors and Response(s) Mediated

B/UV-A receptor	Response(s)
<i>cry1</i> , <i>cry2</i> (for cryptochromes 1 and 2)	De-etiolation, anthocyanin synthesis, flowering
<i>nph1</i> , <i>npl1</i> (for non phototropic hypocotyl, and <i>nph</i> -like)	Phototropism
??	Stomatal opening

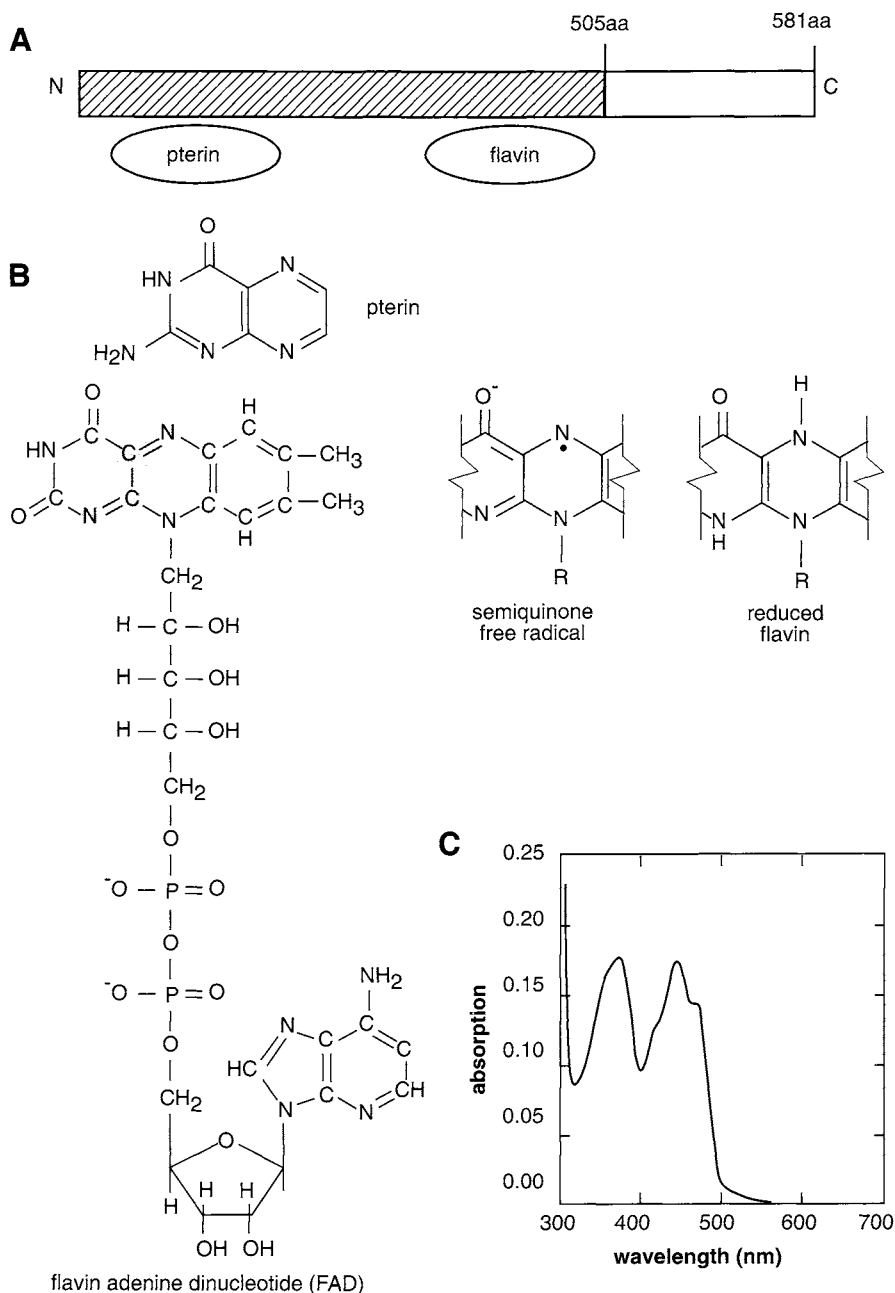


FIGURE 26-13 Structure and absorbance spectrum of cry1. (A) The cry1 apoprotein consists of an N-terminal and a shorter C-terminal domain. The N-terminal domain carries a flavin adenine dinucleotide (FAD) chromophore and likely another chromophore of the pterin type. (B) Structures of a pterin and flavin adenine dinucleotide (FAD), both fully oxidized. In FAD or FMN (flavin mononucleotide), the coordinated double bonds between N-1 and C-10 and between N-5 and C-4 in the isoalloxazine ring participate in the oxidation-reduction reactions. The semiquinone-free radical and fully reduced structures also are shown. (C) The absorbance spectrum of cry1 holoprotein. Adapted from Ahmad *et al.* (1998) and Ahmad and Cashmore (1993).

to bacterial photolyases. Photolyases are enzymes that catalyze the repair of pyrimidine dimers in DNA caused by exposure to UV. They carry either a pterin or a deazaflavin chromophore, which absorbs in UV-A

or blue, respectively, and presumably passes the energy to another chromophore, a flavin adenine dinucleotide (FAD). The FAD is reduced and brings about DNA repair by cleavage of the pyrimidine

dimer. Like photolyases, cry1 has a FAD chromophore. It also carries another chromophore, which is thought to be a pterin; however, unlike bacterial enzymes, cry1 has no photolyase activity. Also, the FAD bound to the CRY1 apoprotein is believed to alternate between the oxidized state and a semireduced, but stable semiquinone form, FADH^\bullet , not the fully reduced FADH_2 . The identity of the chromophore(s) in cry2 is still unknown, although it probably is a flavin. Cry2 also has no photolyase activity.

Cry1 is a soluble protein like phytochromes, and immunological data indicate that it is present ubiquitously in all tissues, cotyledons, hypocotyls, and roots of seedlings. However, unlike phytochromes, where the linear tetrapyrrole is bound covalently to the apoprotein, the flavin chromophore is noncovalently bound and is released completely by heat or acid denaturation of cry1.

Both cry1 and cry2 participate in blue light-induced inhibition of hypocotyl growth and in the expansion of cotyledons; also, their overexpression in transgenic plants shows an exaggerated response in the blue part of the spectrum. However, the two cryptochromes differ in stability, fluence response, and physiological roles. While cry1 is stable in light, cry2 levels decline rapidly in green, blue, and UV-A light (wavelengths that activate the receptor, although not in dark or R light). Unlike *PHYA*, however, *CRY2* transcript accumulation is not affected and it is thought that a protein degradation mechanism is involved.

Mutants specific to *CRY1* or *CRY2* and transgenic plants overexpressing either cry1 or cry2 indicate that the two receptors are active at different fluences of light and mediate specific B light responses. Cry1 is thought to be active at higher fluences of B light and plays a role in setting of the biological clock. In contrast, cry2 seems to be active under low fluence blue light and is involved in the flowering response. A late flowering mutant of *Arabidopsis*, *fha-1*, is allelic to *cry2*.

3. SECTION SUMMARY

Light is one of the most complex and variable environmental signals. It varies in quality, quantity, direction, and duration. Biological responses are triggered by lights of different wavelengths, especially blue and red/far-red wavelengths. They also require different fluences of light: some are triggered by very low fluences; others require higher, but still relatively low fluences; and still others require continuous irradiance for long periods. These responses are mediated via an

array of photoreceptors. Phytochromes are a family of photoreceptors that mediate red/far-red-regulated responses. Cryptochromes are a family of one class of blue/UV-A receptors, which mediate many blue light responses. Other classes of blue/UV-A receptors are also known (see Chapter 27).

Phytochromes occur as dimers in solution. Each molecule has a linear tetrapyrrole-type chromophore attached covalently to an apoprotein. The chromophore is synthesized in the plastid, but the apoprotein, coded by its gene, is translated in the cytoplasm where assembly of the holoprotein occurs. In *Arabidopsis*, five families of genes, *PHYA-PHYE*, encode apoproteins and give rise to holoproteins, phyA through phyE. Other plants have similar families with a variable number of members in each family. All phytochromes show similar (although not identical) absorption spectra with peaks in red and far-red and strong shoulders in blue UV-A regions. The phytochrome molecule cycles between two molecular species. The Pr form absorbs primarily in the red region and is converted to the Pfr form. The Pfr form absorbs primarily in the far-red region and is converted back to the Pr form. The transition from the Pr to the Pfr form and *vice versa* involves intermediates, which are different in the two transitions and which can accumulate in continuous irradiation. The Pfr form is considered to be biologically active, and the Pfr/P ratio is critical for triggering a response. Many phytochrome responses activated at low fluences of light are R/FR reversible, whereas others mediated at very low fluences or high irradiances are not reversible. Phytochrome A is the major phytochrome in dark-grown tissues, but it degrades and its synthesis is stopped in light. Hence, the relative proportions of phyA and light-stable phytochromes, e.g., phyB and phyC, change in the light environment and, although substantial amounts of phyA still remain, phyB and other light-stable phytochromes become more important players.

Cryptochromes also occur as members of small gene families. Two members, cry1 and cry2, are known in *Arabidopsis*, and homologues are being discovered in other plants. Cryptochromes are soluble proteins; they have an apoprotein moiety and chromophores that absorb primarily in UV-A/blue and green regions. Cryptochrome 1 has two chromophores, a pterin-type molecule and a flavin adenine dinucleotide, but the details of changes on blue light absorbance are not clear. Cry1 and cry2 differ in their sensitivities to and stability in light. Cry2 seems to be more active at low fluences of blue light and is less stable than cry1. The two cryptochromes mediate some common and unique responses.

Molecular genetic studies have allowed a dissection of the roles of various phytochromes and cryptochromes in the regulation of red/far-red and blue light responses. This topic is covered in the next section.

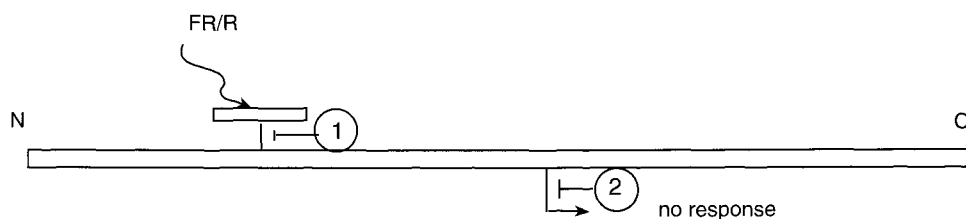
SECTION II. PHYSIOLOGICAL ROLES OF PHYTOCHROMES AND CRYPTOCHROMES

1. ROLE OF MUTANTS IN DEFINING THE FUNCTIONS OF PHOTORECEPTORS AND THEIR SIGNALING PATHWAYS

Since there are many phytochromes and at least two cryptochromes, the challenge has been to decipher which response is mediated by which photoreceptor and whether the same response is mediated by more than one photoreceptor. Two methods have

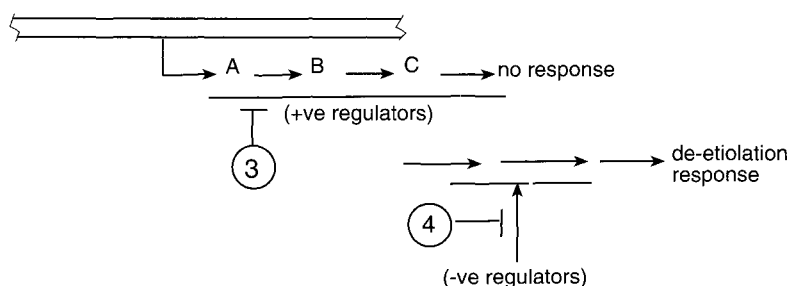
been used: (i) overexpression of a phytochrome or cryptochrome in a transgenic plant and/or (ii) generation of mutants that are defective in a photoreceptor. In both cases, the responses are analyzed under different light regimes. The second approach is by far the more reliable because it obviates some of the disadvantages inherent in overexpression of a gene. In the second approach, mutant selection is based on screens that utilize a specific photomorphogenic response as the criterion. Hence, mutants are selected that are defective in perception of the light signal, as well as those defective in some step of signal transduction.

Figure 26-14 shows diagrammatically that using de-etiolation as a screen, at least four classes of mutants can be isolated. Classes 1–3 are selected by screening for an etiolated phenotype in seedlings grown in white light. This was the original screen used by Maarten Koornneef and associates at Wageningen Agricultural University, Netherlands in 1980 for selection of the first *hy* (for long hypocotyl) mutants in *Arabidopsis*.



Class 1. Defect in synthesis/assembly of chromophore

2. Defect in apoprotein - light signal is perceived, but no response is effected



3. Mutations in signal transduction chain (A, B, C,..., are intermediates in the chain)

4. Mutations that give a de-etiolated phenotype when grown in dark
Negative regulators prevent de-etiolation in the dark

FIGURE 26-14 A schematic illustration of four classes of mutants and their sites of action. Seeds are mutagenized, and seedlings are screened either for an etiolated phenotype in light-grown material (classes 1–3) or for a de-etiolated phenotype in dark-grown material (class 4).

1.1. Chromophore Mutants

Some mutants that show an etiolated phenotype when grown in light could be defective in chromophore synthesis or its linkage with the apoprotein (class 1 mutants; Fig. 26-14). This can be tested easily by supplying the chromophore or its precursors (e.g., biliverdin) exogenously; if the mutants are rescued, they are chromophore mutants. Several chromophore-deficient mutants are known (see Fig. 26-8). These mutants are of limited interest in deciphering the roles of photoreceptors, but they are useful in the study of chromophore synthesis and assembly and have been used in the analysis of signaling pathways.

1.2. Photoreceptor Synthesis Mutants

Another class of etiolated mutants are defective in the apoprotein and show a null or impaired morphogenic response (class 2 in Fig. 26-14). In parallel with the terms used for hormone mutants, these may be referred to as photoreceptor synthesis mutants. They can be further discriminated by being grown in continuous red (Rc), far red (FRc), or blue (Bc) light. As we will see later, far-red responses are mediated exclusively by phyA, whereas responses due to red are mediated primarily by phyB. The major blue light photoreceptor for the de-etiolation response, to date, is cry1. Thus, mutants specifically defective in one or another photoreceptor have been obtained from several plants (Table 26-3). When first isolated, some were named after the screen or the phenotype, but now they are often referred to by

the photoreceptor they are defective in, e.g., *phyA*, *phyB*, and *cry1*. Several mutant alleles in each case are known; some are more severe than others. For use in monitoring the effects on specific photomorphogenic responses, null mutants that lack the apoprotein altogether or produce a nonfunctional protein, and hence are unlikely to be leaky, are preferred. Curiously, all mutant *phyA*, *phyB*, and *cry1* alleles currently isolated are partially dominant over WT alleles.

Not only single mutants, but, increasingly, double and triple mutants (e.g., *phyA phyB* or *phyA phyB cry1*) are being used with greater accuracy for pinpointing the effects of a photoreceptor on a photomorphogenic response.

1.3. Mutants Defective in Signal Transduction

A third class of mutants is defective in signal transduction (class 3 in Fig. 26-14). These mutants have a functional photoreceptor, but there is a lesion in some protein in the signal transduction chain such that the expected response is not effected. When first isolated, these mutants cannot be distinguished from class 2 mutants, but if the mutant gene has been cloned, a sequence comparison immediately establishes whether the mutation is in the apoprotein. If not, the chances are that it is in a protein in the signal transduction chain. Thus, *hy5* is a specific mutation in signal transduction. Some authors refer to these mutations as mutations in the positive regulators of signal transduction. To further discriminate between signaling from

TABLE 26-3 Some Photoreceptor Mutants with Defective Apoproteins (Synthesis Mutants)^a

Screen	Plant	Mutant
Far-red (FRc)	<i>Arabidopsis thaliana</i>	<i>phyA</i> (<i>hy8</i> for long hypocotyl; <i>fre1</i> for far-red elongation; <i>fhy2</i> for far-red long hypocotyl)
	<i>Lycopersicon esculentum</i> (tomato)	<i>fri</i> (for far-red light insensitive)
	<i>Pisum sativum</i> (pea)	<i>fun1</i>
Red (Rc)	<i>A. thaliana</i>	<i>phyB</i>
	<i>L. esculentum</i> (tomato)	<i>tri</i> (for temporarily red-light insensitive)
	<i>P. sativum</i> (pea)	<i>lv</i>
Blue (Bc)	<i>A. thaliana</i>	<i>cry1</i> (<i>hy4</i>)
	<i>A. thaliana</i>	<i>cry2</i>

^aIn addition to those listed here, several putative *phyB* mutants are also known [e.g., *Sorghum* (*ma^R₃*), *Brassica* (*ein*), cucumber (*lh*)]. In *Arabidopsis*, *phyD* and *phyE* mutants are also known.

specific photoreceptors, mutants can be obtained by mutagenizing lines where one photoreceptor is the pre-dominant functional photoreceptor. Among the original *fly* mutations in *Arabidopsis*, *fly1* and *fly3* are specific defects in phyA signaling.

1.4. Mutants That Show a Light-Grown Phenotype When Grown in Dark

A different screen is used to obtain the mutants of the fourth class (see class 4, Fig. 26-14). The screen in this case is selection for a de-etiolated phenotype in dark-grown seedlings. Many mutant loci have been identified using this screen and their wild-type genes

cloned. The encoded proteins are believed to repress de-etiolation of seedlings while in the dark and thus act as negative regulators of the photomorphogenic program in seedling growth.

Among the mutant classes just described, class 2 mutants are most useful in deciphering the roles of photoreceptors in vegetative development and reproduction. Class 3 and class 4 mutants are useful in elucidation of the signaling pathways. They are covered in Section III. In Section II, we continue with the physiological roles of photoreceptors, but before proceeding with that, some important information on the role of phytochromes in R/FR sensing is provided in Box 26-3.

BOX 26-3 PHYTOCHROMES SENSE RELATIVE RED:FAR-RED RATIOS IN THE ENVIRONMENT

THE PFR/P RATIO REGULATES biological responses. As explained earlier, the Pr and Pfr forms of phytochrome exist in a state of dynamic equilibrium because of the absorption spectra of the two forms (see Fig. 26-9). Biological responses are determined by the ratio of phytochrome that exists in the Pfr form to total phytochrome (Φ or Pfr/P), not the quantity of Pr or Pfr. When a certain concentration of Pfr/P is reached, the response is triggered. Phytochrome thus acts as a molecular switch.

Sensing the R:FR ratio in the environment is the fundamental sensory function of phytochrome. Plants use the photoconversion between Pr and Pfr forms to sense the relative amounts of red light (ca. 600–700 nm) and far-red light (ca. 700–800 nm) in actinic radiation. If the photon fluence rate is sufficient to drive the photoconversions to equilibrium, the R:FR ratio in actinic radiation can be used to calculate the Pfr/P ratio in a plant.

Since the amounts of red light and far-red light vary at different times of day and in different ecological situations (Table 26-4), the proportion of a phytochrome that exists at any one time in the Pr vs Pfr form also varies. This change is used by the plants to sense the prevailing environmental conditions, including the proximity of neighbors.

TABLE 26-4 Ecologically Important Light Parameters^{a,b}

	Photon flux density ($\mu\text{mol m}^{-2}\text{s}^{-1}$)	R/FR
Daylight	1900	1.19
Sunset	26.5	0.96
Moonlight	0.005	0.94
Ivy canopy	17.1	0.13
Some lakes at a depth of 1 m	300–1200	1.2 to 17.2
At a depth of 5 mm in soil	8.6	0.88

^aThe light intensity factor (400–800 nm) is given as the photon flux density, and light that can be used by phytochrome is given as the R/FR ratio. Absolute values are taken from spectroradiometer scans; values should be taken to indicate relationships between the various natural conditions and not as actual environmental means.

^bReprinted with permission from Smith (1982), © Annual Reviews.

Plants use the various phytochromes to accurately sense and respond to light intensities over 7 to 8 orders of magnitude (see Table 26-1). At the low end, VLF responses are saturated at fluences as low as 0.1 nmol m^{-2} . At these levels of light, no more than 2% of total phytochrome can be converted to Pfr, or Pfr/P is about 0.02. Since under saturating FR the ratio of Pfr/P is still about 3% (~ 0.03), VLF responses are not photoreversible. So far they are known to be mediated exclusively via phytochrome A and are characterized only on the basis of their action spectrum.

2. ROLE OF PHOTORECEPTORS IN PHYSIOLOGICAL RESPONSES

The availability of *phy* and *cry* mutants and over-expression of one or another photoreceptor in a transgenic plant have allowed a dissection of the roles of individual photoreceptors in complex physiological and ecophysiological processes with a specificity that was impossible until recently. So far, these studies have been mostly centered on defining the roles of *phyA*, *phyB*, and *cry1*, although other phytochromes and *cry2* are also beginning to be examined. In the following, the roles of these photoreceptors in seed germination, de-etiolation, and shade avoidance responses are highlighted. The phototropic response is covered in Chapter 27. The emphasis in this section is on seed plants, especially angiosperms. Many responses in cryptogams are also mediated by phytochromes and blue light receptors. They are covered briefly in Box 27-1 in Chapter 27.

2.1. Germination of Seeds

It has been known for a long time that seeds of many herbaceous, undomesticated, or pioneer species require light for germination. These seeds are usually small with little food reserves, with attendant danger that their seedlings might not reach the surface and light before the food reserves are used up. Germination of these seeds is strongly dependent on a light signal, and it is believed that the phytochromes enable these seeds to detect their proximity to soil surface and, thus, time their germination.

Light-induced seed germination typically shows a biphasic fluence response curve (Fig. 26-15). Some seeds germinate at very low fluences of light when the calculated Pfr/P ratio is $\sim 0.1\%$, whereas others germinate in increasing numbers as the fluence is increased and the Pfr/P ratio gets above 1% (LF range).

Seeds need to be wet to perceive the light signal, but they can be desiccated after the light treatment, dried, and stored for months, and they remember the last

light treatment. For example, lettuce seeds, which are imbibed, exposed to R light, and then desiccated, germinate after a year or two without any subsequent R treatment. In contrast, seeds given FR during imbibition and dried remain dormant. This is because both Pr and Pfr are stable in the dry state. In the field, the seeds probably remember the light they received at the time of maturation/desiccation (see Chapter 18). Since R:FR ratios in the natural environment, as well as within individual fruits, vary at the time of seed maturation, the variability in Pfr/P ratios of individual seeds can be enormous.

Which phytochrome regulates seed germination? Experiments done with *Arabidopsis phy* mutants provide the answer. It should be kept in mind that the *phyA* mutant means *phyA* is disabled, the response is due to *phyB*; the *phyB* mutant, in contrast, is used to interpret the action of *phyA*.

2.1.1. Seed Germination in *Arabidopsis*

Seed germination in *Arabidopsis* has been studied using *phyA* and *phyB* mutants under different light regimes and fluences. As shown in Fig. 26-16, under continuous white light, seeds of all three genotypes germinate nearly 100% within 3 days. However, in

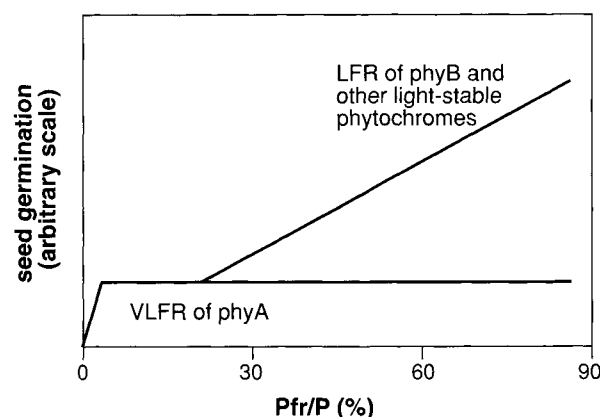


FIGURE 26-15 Biphasic response of Pfr/P in seeds. Seeds germinate both under very low fluence and in increasing numbers under low fluences. Adapted with permission from Casal *et al.* (1997), © Blackwell Science Ltd.

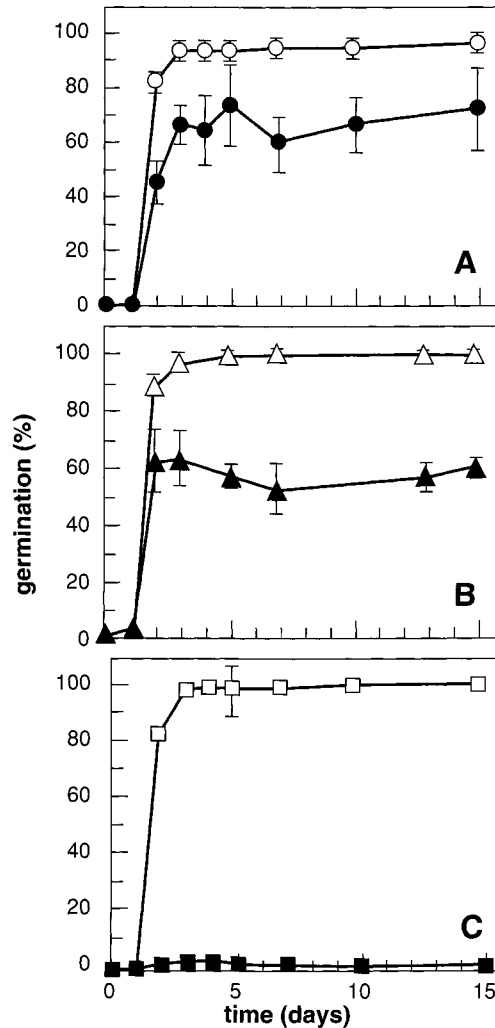


FIGURE 26-16 Seed germination in WT (A) and *phyA* (B) and *phyB* (C) mutants of *Arabidopsis* under continuous white light (open symbols) or darkness (closed symbols). Dormant seeds were plated on nutrient-rich agar and allowed to imbibe for 15 days at $25 \pm 1^\circ\text{C}$. Germination was scored daily and plotted as a percentage of those plated. Values are means \pm SE from at least five determinations. From Shinomura *et al.* (1994).

the dark, *phyB* seeds do not germinate even after 15 days, whereas the WT and *phyA* mutant seeds show substantial germination after 3 days. These data suggest that *phyB* regulates seed germination in the dark, and it probably does so as *phyB* stored in the seed in the Pfr form (PfrB).

That seed germination in the dark is mediated by *phyB* in the Pfr form is shown by giving short pulses of R or FR light to imbibed seeds (Table 26-5). Under all light regimes, *phyB* mutants germinate poorly, whereas *phyB* in the WT or *phyA* background elicits good germination under R light. Moreover, the R-induced response is reversed by FR. This is the typical R/FR reversal phytochrome response (see also Fig. 26-5), and data clearly show that *phyB*, not *phyA*, is involved.

To further analyze *phyA*- and *phyB*-mediated seed germination, seeds imbibed in the dark were given a FR exposure at 1 h, followed by red light at 667 nm for 3 h, and then kept in the dark for 7 days and germination scored. In an alternate protocol, R light exposure was given much later, at 48 h, and germination was scored after 7 days. The difference between the two protocols is in the length of the dark period between FR and R treatments (see Fig. 26-17A).

The percentage germination of WT, *phyA*, and *phyB* seeds is plotted against photon fluence in red light in Fig. 26-17B. From data for WT and *phyB* mutant, it is clear that *phyA* is responsible for germination under very low R light fluence, whereas data for the *phyA* mutant and WT show that *phyB* controls the germination under low fluence ($100\text{--}1000 \mu\text{mol m}^{-2}$).

TABLE 26-5 Germination of *Arabidopsis* WT, *phyA*, and *phyB* Seeds Exposed Briefly to Red (R) and/or Far-Red (FR) Light^{a,b}

Light treatment	Germination (%)		
	WT	<i>phyA</i>	<i>phyB</i>
D	66 ± 10	60 ± 3	1 ± 2
FR	3 ± 1	2 ± 1	2 ± 1
R	90 ± 2	70 ± 5	2 ± 2
R/FR	1 ± 1	6 ± 2	3 ± 1
R/FR/R	90 ± 1	71 ± 13	3 ± 1

^aDormant seeds were plated on nutrient-rich agar, imbibed for 1 h, exposed to R or FR light (0.3 W m^{-2}) for 5 min, and kept in the dark for 4–6 days until scored for germination. Values are means ± SE for at least five determinations.

^bAdapted from Shinomura *et al.* (1994).

In further experiments, it has been shown that the VLF response is not exclusive to red light; indeed any wavelength between 300 and 780 nm can be effective, but the VLF response is mediated exclusively by *phyA*, and not *phyB* (or other light-stable phytochromes).

Experiments where a FR (726 nm) exposure after 1 h is followed at 3 or 48 h by another FR exposure show that *phyA* controls seed germination in WT or a *phyB* background under continuous far red and that it is a high irradiance response. This conclusion is confirmed by the fact that *phyA* seeds show little or no germination under FRc, whereas WT or *phyB* seeds show 60–100% germination, respectively (Fig. 26-18).

The respective roles of *phyA* and *phyB* are supported by genetic crossing or by overexpressing *PHYA* or *PHYB* cDNAs in transgenic tobacco or *Arabidopsis*. For example, a cross between WT and *phyA* mutant yields 25% of the progeny as *phyA/phyA*, which show much reduced germination in FRc. Similarly, seeds of transgenic tobacco or *Arabidopsis* plants overexpressing *PHYB* cDNA show high germination frequencies in the dark compared to seeds from untransformed plants.

In summary, seed germination in *Arabidopsis* is controlled by both *phyA* and *phyB* and can be obtained under varied light fluences. *PhyA* is active under very low fluence red light; it also promotes seed germination under continuous FR, an HIR response. Both of these modes of control are irreversible. *PhyB* controls seed germination in the dark *via* its Pfr form (PfrB). This requires low fluence red or white light and is reversible by FR.

These experiments using *Arabidopsis* mutants help explain how different phytochromes may regulate seed germination in the field.

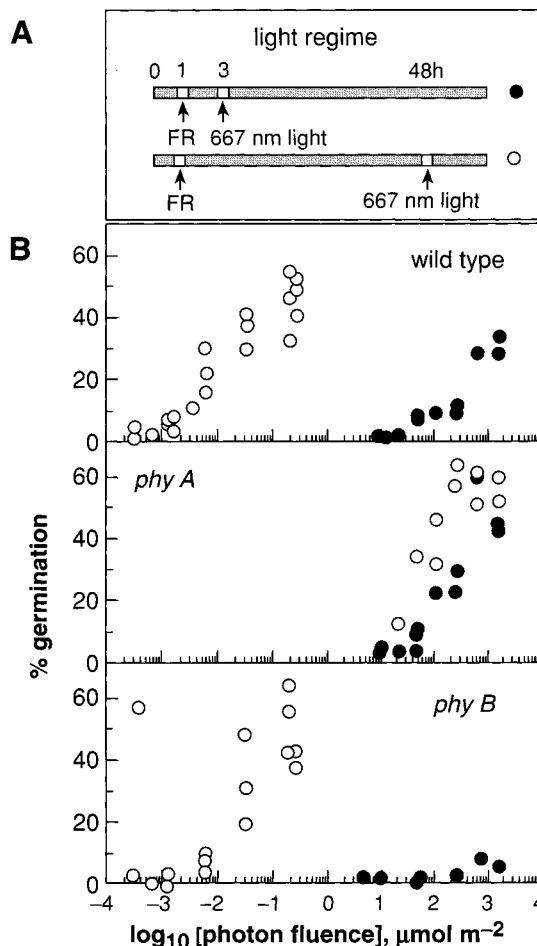


FIGURE 26-17 Effects of incubation time and photon fluence of red light (667 nm) on seed germination. (A) Light regime of the experiment. Gray bars, incubation period on aqueous agar plates in darkness at $25 \pm 1^\circ\text{C}$; white bars with arrows, pretreatments with FR light and exposure to R light (667 nm). (B) Fluence response relationships for WT, *phyA*, and *phyB* seeds. Closed and open symbols are germination rates of seeds that were kept in darkness for 3 or 48 h, respectively. From Shinomura *et al.* (1996) with permission.

2.1.2. Seed Germination under a Forest Canopy or Freshly Disturbed Soil

Forest or cultivated soils commonly retain large banks of dormant seed of pioneer or weed species, respectively, much of which is present at or near the surface. Since *phyA* perceives very low fluences of light that other phytochromes cannot distinguish from darkness, *phyA* is the phytochrome believed to be responsible for light-induced seed germination under a dense forest canopy or under freshly disturbed soil. A dense canopy restricts R and shorter wavelengths from reaching the ground; only a small shoulder in green (550 nm) and a larger shoulder in FR (above 730 nm) reach the ground (Fig. 26-19). Under these

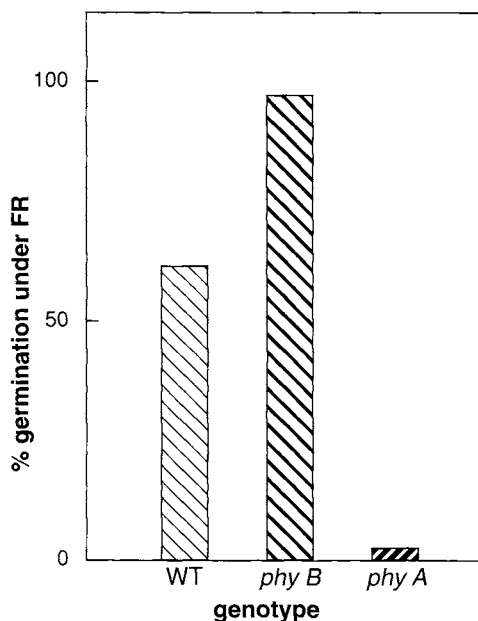


FIGURE 26-18 Seed germination in *Arabidopsis* under continuous far-red light. Adapted from Reed *et al.* (1994).

conditions, seeds maturing at the forest floor are likely to have very little total phytochrome in Pfr form and

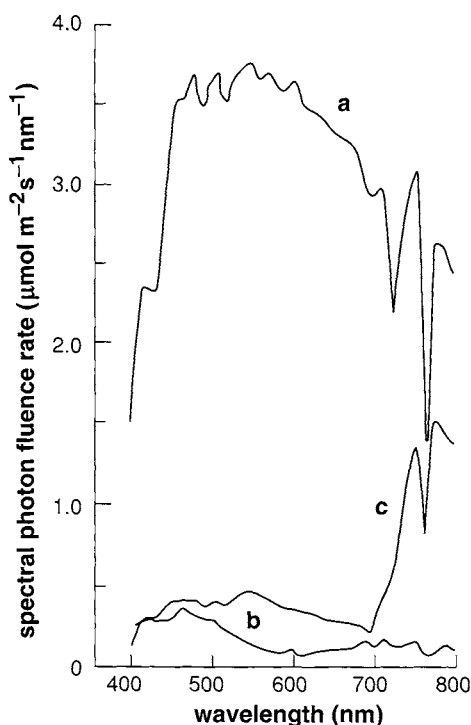


FIGURE 26-19 Characteristic spectral photon distribution of natural radiation. (a) Normal daylight, clear sky; (b) at the point of sunset; and (c) beneath a vegetation canopy. Data obtained with gamma spectroradiometer in July 1981 at Leicester, United Kingdom. From Smith and Morgan (1983).

require R for germination. As mentioned earlier, VLF responses are typically saturated at Pfr/P ratios of 0.1%, and the little amounts of light reaching the forest floor and perceived by phyA are sufficient to raise the Pfr/P ratio above the threshold required. That phyA is involved is confirmed by experiments in which a single pulse of light simulating canopy shade has been shown to cause germination in WT but not in *phyA* seeds of *Arabidopsis*.

Soil disturbance usually provokes seed germination. A large number of weed seedlings usually emerge after daytime than after nighttime plowing, suggesting that light perception *during* disturbance is important. Exposure to light during plowing may be very brief, but some weed seeds acquire extreme sensitivity to light when buried for several months in the field. *Datura ferox* is an aggressive annual weed in temperate and subtropical South America. Seeds produced in summer and autumn lie dormant on or below the soil surface and are triggered to germinate the following spring or summer when soil is turned for cultivation. Fluence-response curves for *D ferox* seeds indicate that during burial there is a shift of 4 orders of magnitude in sensitivity to light, equivalent to a shift from a LF to a VLF response mode; and further that the light-induced promotion of germination of *Datura* seeds is a VLF response. It is suspected that this response is mediated via phyA. What causes the increase in sensitivity to light is unknown, but in laboratory experiments, a positive response to very low fluences of light is often correlated with a long dark period preceding the VLF signal. Increased sensitivity may result from a buildup of the concentration of phyA in Pr (PrA) in the dark period, as shown below, or from the phenomenon of "adaptation" (see Chapter 27)

Long dark period \rightarrow [PrA] increased \rightarrow enhanced sensitivity to light

2.1.3. Colonization of Canopy Gaps

While phyA mediates seed germination under the canopy via a VLF response, phyB and other light-stable phytochromes are believed to mediate seed germination under low fluence R in gaps that appear in the canopy. Some seeds respond almost instantaneously to an altered R/FR ratio, whereas others require a sustained high R:FR ratio, as obtained by several hours of direct sunlight per day for several days, for germination. It is also possible that the VLF response stimulus established by phyA when the seeds were under the canopy, or when the soil was disturbed, would allow germination when the canopy is removed.

Seed germination in tree species is very little investigated, but it is known that some tree seeds in the Pacific northwest of the United States and Canada require open gaps in the canopy to germinate (e.g., Douglas fir, *Pseudotsuga menziesii*), whereas others can germinate under a dense forest canopy (e.g., Mountain hemlock, *Tsuga mertensiana*).

Seed germination is affected not only by light, but also by temperature and hormones, especially gibberellins (GAs). Although the mechanism is not understood, many red light-responsive seeds are induced to germinate at colder temperatures and are inhibited at higher temperatures. Gibberellin regulation of seed germination is covered later in this chapter (see Section IV).

2.2. De-etiolation Response in Light

The de-etiolation response of dicot seedlings is a complex of many responses, which include an inhibition of elongation growth of stem (hypocotyl/epicotyl), opening of the cotyledons (or leaves) and their expansion, differentiation of etioplasts into chloroplasts, and concomitant expression of photosynthetic genes (e.g., *CAB*, *RBCS*). In addition, with increasing light intensity, anthocyanins are accumulated in vacuoles as a partial defense against solar radiation, and many genes involved in flavonoid biosynthesis (e.g., *CHS*, *DFR*) are expressed. Although these responses go together, not all of them are necessarily brought about to the maximal extent under any one treatment or regulated by any one photoreceptor, which suggests that signaling pathways for de-etiolation are branched and regulated by multiple photoreceptors. Moreover, a specific response may be more affected by one photoreceptor than by others, indicating some degree of primacy among receptors for that response.

2.2.1. Inhibition of Hypocotyl Elongation

The inhibition of hypocotyl growth and unfolding and expansion of cotyledons, like seed germination, can be initiated under very low fluences of light, and continue under low fluence or high irradiance response modes. De-etiolation under VLF is regulated exclusively by phyA, whereas phyB is the principal phytochrome involved in low fluence responses. The de-etiolation response in HI mode is mediated in a seemingly redundant manner by each of the three receptors, phyA, phyB, and cry1, as shown in Fig. 26-20. PhyA, phyB, or cry1 are the principal receptors mediating de-etiolation under continuous FR, R, or B lights, respectively. However, under continuous B light, phyA and phyB also play important roles.

2.2.2. Induction of Gene Expression

Hypocotyl growth inhibition, hook opening and unfolding, and expansion of cotyledons are complex responses. The expression of photosynthetic and/or anthocyanin biosynthesis genes, in contrast, is relatively simple and more direct. Like hypocotyl growth inhibition, these genes are expressed in white, red, or blue light, but their induction varies with the developmental context. In etiolated seedlings, both photosynthetic and anthocyanin biosynthesis genes are regulated by white, R, FR, and B lights, but in the leaves of more mature plants, anthocyanin genes are regulated exclusively by blue light.

2.2.2.1. Chloroplast Development and Expression of Photosynthetic Genes

In the light, etioplasts differentiate into chloroplasts, chlorophyll is formed from protochlorophyllide, and photosynthetic genes (e.g., *CAB*, *RBCS*) are expressed. Studies using *phyA* and *phyB*, single and double, mutants indicate that both phyA and phyB promote chloroplast development and expression of *CAB* genes by a brief R light pulse. Single *phyA* or *phyB* mutants respond equally well to R light, but the double mutant shows a much attenuated response, which indicates that either phyA or phyB can mediate these responses (Fig. 26-21). Moreover, phyA mediates *CAB* mRNA induction by FR as well, as it does seed germination and inhibition of hypocotyl elongation.

2.2.2.2. Anthocyanin Accumulation

Single mutants, *phyA*, *phyB*, and *cry1* and some double mutants show that anthocyanin accumulation is severely depleted in continuous R in all mutants, especially in *phyB* and the two double mutants, *hy1 phyB* and *phyA phyB* (Fig. 26-22). Under continuous B light, the wild type shows much more anthocyanin accumulation than under continuous R light, which confirms the importance of blue light in anthocyanin production. Under B light, the accumulation of anthocyanin is severely reduced in *cry1*, *phyB*, and the two double mutants (*hy1 phyB* and *phyA phyB*), which indicates clearly that under blue light, both *cry1* and *phyB* play a role in anthocyanin accumulation. The *phyA* mutant shows intermediate values, which indicates that phyA is not the major mediator of anthocyanin production under blue light. However, under continuous farred (FRc), phyA is the major phytochrome responsible for anthocyanin accumulation.

2.3. From Seedlings to Adult Plants

The dual effect of phytochromes on the suppression of elongation growth and the promotion of cotyledon/

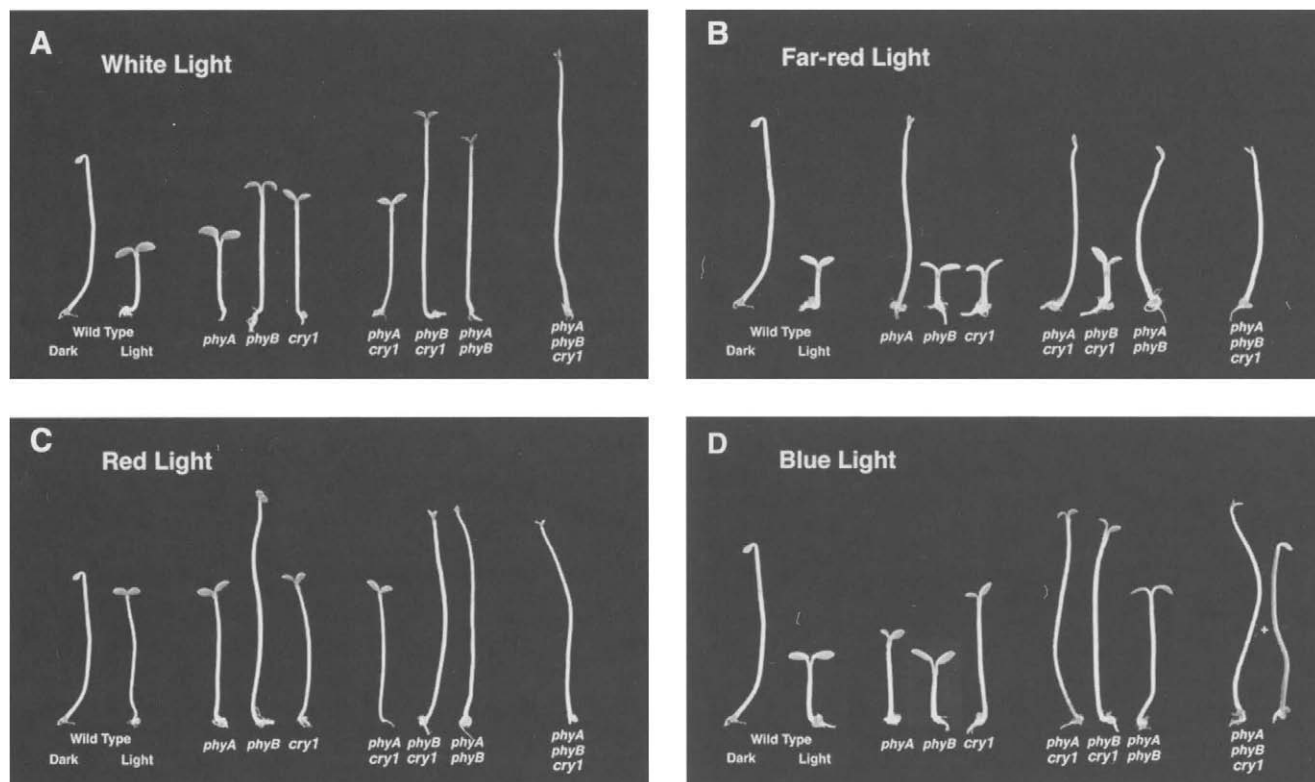


FIGURE 26-20 De-etiolation response of *Arabidopsis* WT, *phyA*, *phyB*, *cry1* single, double, and triple mutants under different but continuous light regimes. In continuous W, all three single mutants show inhibition of hypocotyl growth and opening of cotyledons and their expansion, indicating that in the absence of one, the other two receptors are able to affect de-etiolation. In continuous FR or R, *phyA* or *phyB* mutants, respectively, are etiolated, indicating that *phyA* mediates the de-etiolation response in FRc, whereas *phyB* is the principal receptor mediating de-etiolation in Rc. In continuous B, the *cry1* mutant is partially de-etiolated, which suggests that, in addition to *cry1*, *phyA* and/or *phyB* also participates in the de-etiolation response under B light. The double and triple mutants provide additional information. In Wc or Rc, the *phyA cry1* double mutant shows partial de-etiolation, which indicates that *phyB* plays a major role in de-etiolation under Wc or Rc. In FRc, *phyB cry1* is the only combination that shows de-etiolation; both *phyA phyB* and *phyA cry1* show an etiolated phenotype, which confirms that *phyA* is the principal mediator of the response to FRc. In Bc, *phyA cry1* and *phyB cry1* show an etiolated phenotype, which suggests that although *cry1* is the principal B light receptor, *phyA* and *phyB* also play a role in de-etiolation under B light. Moreover, because the double mutant *phyA phyB* shows de-etiolation, it means that *cry1* mediates de-etiolation in a *phyA phyB* null mutant background. The triple mutants, under all light regimes, show an etiolated phenotype, which confirms that all three receptors have a role in de-etiolation. Seeds were germinated on nutrient-rich agar plates for 4 days in dark to ensure even germination and were transferred for 24 h to light before being transferred to continuous white light, far-red light, red light, or blue light for another 5 days. Light fluence in each case was $50 \mu\text{mol m}^{-2} \text{s}^{-1}$. Photographs were taken at the end of 5 days From Neff and Chory (1998).

leaf expansion (photosynthetic capacity) at the seedling stage continues into adult life, but the relative importance of different phytochromes seems to change. Since *phyA* perceives very low fluences of light, fluences that other phytochromes are blind to, de-etiolation of seedlings in the first instance is mediated via *phyA*; later as seedlings reach more open light, other phytochromes, which are light stable, take over. The latter phytochromes essentially mediate a low fluence response in red light. *PhyA* is also able to mediate a low fluence response to red light, although its relative contribution in open light may be

less. Both *phyA* and the light-stable phytochromes also mediate their effects *via* the high irradiance response mode in continuous FR and red light, respectively.

A dramatic illustration of light acting as a morphogen to regulate plant growth is shown in Fig. 26-23. Potato plants were transformed by a construct of *Arabidopsis PHYB* cDNA under a constitutive promoter. Plants overexpressing *phyB* show much reduced height, enhanced lateral branching, and enhanced photosynthetic capacity (increased greening) than the untransformed control.

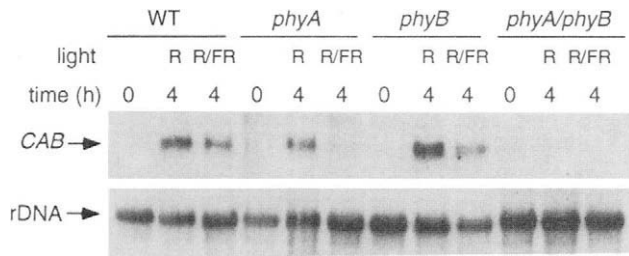


FIGURE 26-21 Induction of *CAB* mRNA in etiolated wild-type, *phyA*, *phyB*, and *phyA phyB* double mutant seedlings by a flash of red light. Seedlings were grown for 5 days in the dark, given a pulse of red (R) light or red followed by far-red (FR) light, and then returned to darkness for 4 h until extraction of RNA. A *CAB* cDNA and a ribosomal protein cDNA (control) were used as hybridization probes. From Reed *et al.* (1994).

2.4. Proximity Perception and Shade Avoidance

Plants have evolved two basic strategies to deal with shade by neighboring plants; tolerate it or avoid it. Shade-tolerant species grow well in shade, whereas others, adapted to open, sunny areas, are intolerant of shade. Plants of shade-intolerant species show a syndrome of responses, known as shade avoidance responses, when other competing plants are near by. These responses include apical growth and elongation of internodes and petioles, suppression of lateral branching, suppression of leaf expansion and thickness, and early flowering.

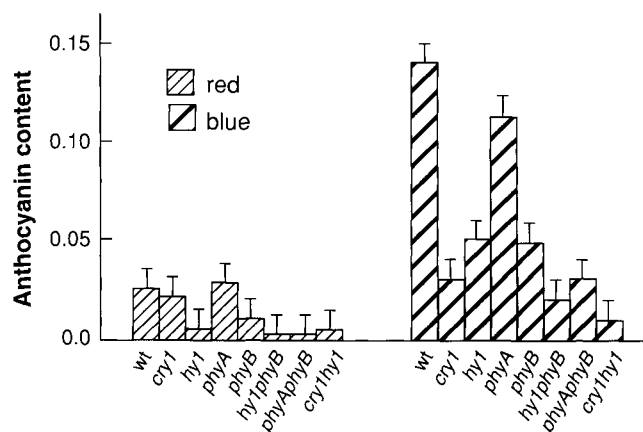


FIGURE 26-22 Anthocyanin accumulation in phytochrome- and cryptochrome-deficient single and double mutants of *Arabidopsis* under continuous red or blue light; *hy1* is a chromophore (P Φ B) mutant. Anthocyanin content is expressed as $A_{530} - 0.25A_{657}$ to correct for the absorption of chlorophyll. Error bars represent mean SE among three independent experiments. The fluence rate for red and blue light is 40 and 36 $\mu\text{mol m}^{-2} \text{s}^{-1}$, respectively. Reprinted with permission from Ahmad and Cashmore (1997), © Blackwell Science Ltd.

Shade-intolerant plants perceive proximity to neighbors by monitoring a specific signal, the R:FR ratio, and not simply a reduction in the level of irradiance. This is shown in the laboratory or greenhouse by growing a shade-intolerant species under a neutral density filter, which reduces the level of photosynthetically active radiation (PAR), without affecting any particular wavelength, or growing the same species under a light regime with altered R:FR ratios while maintaining the same PAR (Fig. 26-24). As shown in Fig. 26-24B, a reduction in the R:FR ratio stimulates the syndrome of shade avoidance responses. In a natural environment, the R:FR ratio is modulated by light that is reflected from, or filtered through, vegetation. A decline in the R:FR ratio is perceived by shade-intolerant species as indicative of a proximity to neighbors.

Results from an experiment on internodal elongation in *Chenopodium album* seedlings grown under varying ratios of R:FR light are shown in Fig. 26-25A. A low R:FR ratio results in a lower concentration of the biologically active Pfr form relative to total phytochrome, which is a signal for shade avoidance responses to be initiated. A plot of internodal elongation against Pfr/P ratios shows an inverse relationship between the Pfr/P ratio and the rate of stem elongation (Fig. 26-25B). Shade-intolerant species show a much steeper slope than shade-tolerant species, which are relatively unaffected by R:FR ratios. Not only plants of different species, but also plants of the same species adapted to different ecological conditions (ecotypes) show variations in their capacity for shade tolerance. Thus, populations of *Impatiens capensis* or *Satureja douglasii* growing in open habitats are more shade intolerant than populations growing in woodlands or under shade.

Shade avoidance responses are difficult to measure in the field, but they can be partially phenocopied in the laboratory or greenhouse by giving plants a 20 to 30-min pulse of FR at the end of a normal day (EOD) just before darkness or by supplementing the normal photoperiod with pulses of FR. The EOD FR is R reversible, even if given several hours later. Both the shade avoidance response and the EOD FR are mediated principally by phyB. However, evidence is accumulating that phyB is not the only light-stable phytochrome mediating shade avoidance responses. The *phyB* single and *phyA phyB* double mutants of *Arabidopsis*, as well as some *phyB*-type mutants in other plants (e.g., *lh* cucumber), still show enhanced elongation in response to EOD FR or supplemental FR, which suggests strongly that other phytochromes in addition to phyB mediate shade avoidance responses.

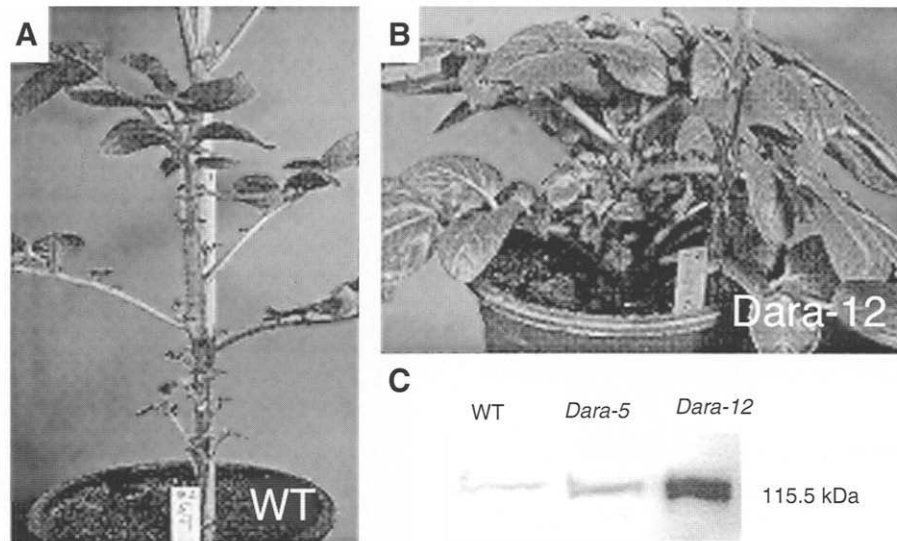


FIGURE 26-23 Phenotypes of a representative wild-type (WT) potato (A) and a 5-week-old potato plant overexpressing phyB (Dara-12) (B). Both plants were grown under moderate light conditions ($0.15\text{--}0.5\text{ mmol m}^{-2}\text{ s}^{-1}$) in a greenhouse. (C) Expression levels of phyB in WT, Dara-12, and an intermediate overexpressor (Dara-5) by immunoblotting using a phyB-specific monoclonal antibody. From Thiele *et al.* (1999).

A *phyE1* mutant of *Arabidopsis* has been isolated from a parental stock that was null for both *phyA* and *phyB* (*phyA phyB* double mutant). The *phyE1* mutant shows highly elongated internodes and reduced leaf blade area compared to the rosette habit

of the parent plant (Fig. 26-26), which suggests that *phyE* also participates in the suppression of internodal elongation in light.

The phytochrome-mediated regulation of plant growth is of adaptive value in the natural environment.

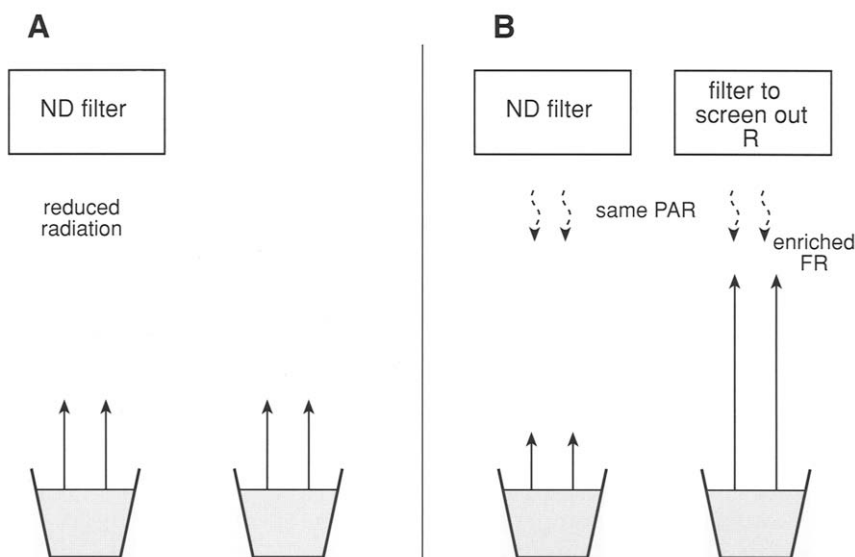


FIGURE 26-24 Diagrammatic representation of plants grown under reduced light intensity or under a reduced R:FR ratio. (A) Plants grown under a neutral density (ND) filter (left) receive less radiation than the ones on the right, but their heights are approximately the same. (B) The two sets of plants are grown under the same PAR, but the one on the right receives more FR.

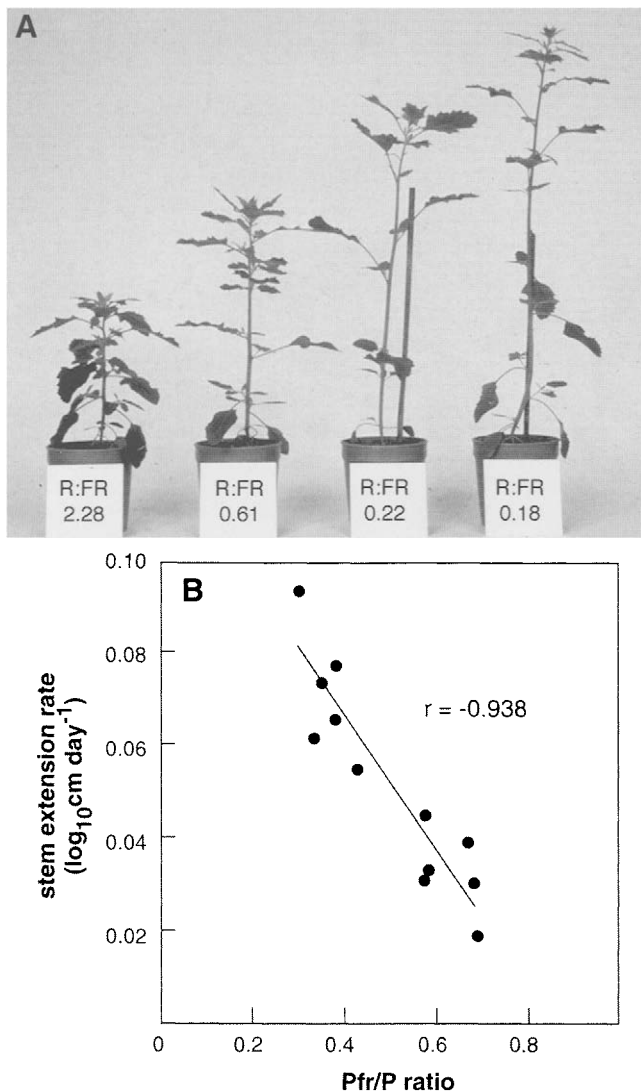


FIGURE 26-25 Internodal elongation as a function of R:FR ratio in actinic radiation. (A) Appearance of *Chenopodium album* seedlings after a 15-day treatment with white light sources of varying R:FR ratios. Prior to transfer to spectral quality cabinets, the seedlings had all been grown in white light having a R:FR of 2.28 for 21 days. (B) Linear relationship between phytochrome photoequilibrium (estimated from the R:FR ratio of the incident radiation) and logarithmic growth rate of seedlings in A. Modified from Smith and Morgan (1983) and Courtesy of Garry Whitelam, University of Leicester, Leicester, UK.

Plants in which *phyB* is disabled show a constitutive shade avoidance response. They do as well as the wild type when crowded, but suffer serious competitive disadvantage when grown in an open environment because they cannot stop their elongation and set fewer seeds. In contrast, *phyA* mutants are unable to stop elongating in a FR-rich environment under a dense canopy and die.

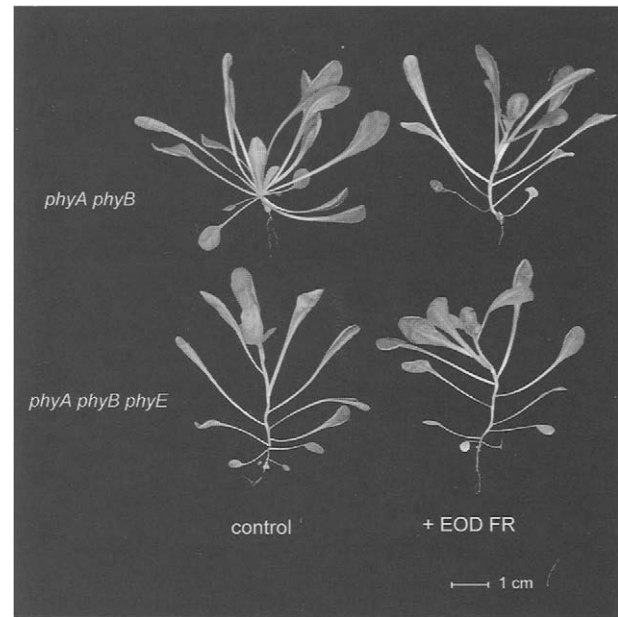


FIGURE 26-26 Phenotypes of *phyE1* and *phyA phyB* double mutant (control) of *Arabidopsis*. Plants were grown for 60 days under 8-h light and 16-h dark cycles. From Devlin *et al.* (1998).

The availability of single, double, and triple mutants for phytochromes and cryptochromes is allowing an analysis of interactions between different photoreceptors. Thus, it has been shown that *phyD* can partially substitute for the loss of *phyB* in the inhibition of hypocotyl elongation, but only if both *cry1* and *phyD* are present.

3. SECTION SUMMARY

Seed germination and the syndrome of responses known as de-etiolation (in dicot seedlings) and shade avoidance are regulated primarily by phytochromes. Certain aspects of de-etiolation, such as inhibition of stem growth, unfolding and expansion of cotyledons, and anthocyanin production, are also regulated by cryptochromes. Analysis of monogenic single mutants, *phyA*, *phyB*, and *cry1*, and also double and triple mutants, coupled with overexpression of one or another photoreceptor in transgenic plants, has revealed the specific roles of phytochromes and *cry1* with a specificity hitherto unknown. Among the phytochromes, *phyA* is the only one that mediates the responses just given under very low fluences of light. It is active over a broad spectrum of VLF light, including wavelengths in UV, blue, green, red, and near far-red. These *phyA*-mediated responses to VLF

light are not photoreversible. PhyA is also active under continuous FR (HIR FR). These same responses are induced by phyB and, most likely, other light-stable phytochromes under low fluence red light (~ 1000-fold more light) given as a pulse or continuously (R:FR reversible or HIR mode). Inhibition of hypocotyl elongation, expansion of cotyledons, and anthocyanin synthesis in seedlings under continuous blue light (HIR B) are mediated by cry1 in concert with phyB. Among the light-stable phytochromes, there is increasing evidence that, in addition to phyB, phyD, phyE, or their homologues, they are involved in shade avoidance and flowering responses of adult plants.

The phyA-mediated responses in the FR HIR mode seem to imply that the response is brought about by the Pr form, which is generally considered biologically inactive. As an explanation, it has been suggested in the past that it is not the newly synthesized PrA in the dark, but rather the recycled PrA that has undergone repeated photocycles that is involved. This explanation is no longer considered valid. In a detailed study on inhibition of hypocotyl elongation by phyA operating in the HIR mode, it has been suggested that a short-lived signal induced during the photoconversion of PfrA to PrA provides the biological response. This mode of photoperception by phyA for HIR is essentially different from that by phyA for VLFR (or LFR) and phyB for LFR, which involve conversion of Pr to Pfr.

The fact that phyA, phyB, and cry1 can bring about inhibition of epicotyl/hypocotyl elongation in white light has been sometimes referred to as functional redundancy. That statement needs to be qualified. Cell/organ growth, as shown in Chapter 15, is a highly complex phenomenon involving water uptake, acidification of the apoplast, wall loosening by a battery of enzymes, cytoskeletal organization, and synthesis of membranes, polysaccharides, and proteins. In order to consider something as functionally redundant, it must be shown that phyA or phyB or cry1 inhibits cell/organ growth at the same locus. Similarly, the synergism between phyB and cry1 in the inhibition of hypocotyl/epicotyl elongation in continuous blue light may be evidence of an interaction between the two receptors. Alternatively, the two receptors may be targeting different facets of the same phenomenon, i.e., inhibition of growth. Our current information cannot resolve these issues. The roles of various photoreceptors also change with the developmental state of the plant. For instance, accumulation of anthocyanins in the seedling stage is regulated by phyA, phyB, and cry1, but in the adult plant or mature leaves it is regulated primarily by blue light.

SECTION III. SIGNAL TRANSDUCTION

1. SIGNALING BY PHYTOCHROMES

How do phytochromes and cryptochromes bring about these responses? As far as cryptochromes are concerned, there is as yet little information other than the fact that the perception of blue light involves a change in the redox status of the flavin moiety. Much more is known about phytochromes, but the available information, although enormous and increasing, is still like single unconnected pieces in a jigsaw puzzle. Moreover, our information still derives mostly from phyA and phyB, and their mutants. Any proposed scheme therefore is at best tentative and is likely to change in the future as we know more about the other pieces in the puzzle and other photoreceptors.

1.1. Domains Important in Phytochrome Signaling

The absorption of red or far-red light causes reversible changes in the chromophore (phytochromobilin), accompanied by conformational changes in the apo-protein, and culminates in the transfer of the signal to the first recipient in the signal transduction chain. Site-directed mutagenesis in *PHY* genes and deletion constructs of *PHYA* or *PHYB* cDNAs expressed in transgenic plants have revealed the domains of the phytochrome molecule that are likely participants in signaling.

As mentioned in Section I, deletion of the C-terminal half yields adducts that are spectrally active, but are unable to dimerize or to affect a biological response. Further dissection of the C-terminal region indicates that missense mutations in phyA and phyB molecules are clustered in a stretch of about 160 amino acids in the middle, at the beginning of the C-terminal half. This region partly overlaps at both ends by two direct repeat amino acid sequences, known as PAS1 and PAS2. The 40 amino acid PAS motif (named after the *Drosophila* period clock protein PER, the mammalian aromatic hydrocarbon nuclear translocator ARNT, and *Drosophila* single-minded protein SIM) is being found in a growing family of regulatory proteins of diverse functions. This region is referred to here as PAS-related domain or PRD (Fig. 26-27).

The PAS domain is important because intermediates in early steps of phyA and phyB signaling (e.g., PIF3 or NDPK2, see later) do not interact with phyA mutated in one of the PAS domains.

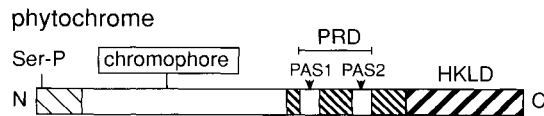


FIGURE 26-27 A schematic diagram of higher plant phytochromes indicating domains considered important in signaling. A short stretch at the N-terminal end and two adjacent regions in the C-terminal half—a PAS-related domain (PRD) and a histidine kinase-like domain (HKLD)—are shown. Serine-7 in the N-terminal domain is shown as phosphorylated. Adapted from Elich and Chory (1997) with permission from Elsevier Science.

The PRD is adjacent to the C terminus region, which bears sequence similarity to the histidine kinase domain of the sensor proteins in the prokaryotic two-component signaling systems (see Box 21-1 and Fig. 21-7 in Chapter 21). This region is referred to as the histidine kinase-like domain (HKLD, see Fig. 26-27). Both PRD and HKLD may be functionally related (see later).

The serine-rich region near the N-terminal, although not essential, is needed for the modulation of biological activity. The serine residues, especially Ser-7, have been shown to be phosphorylated *in vivo*. Moreover, replacement of the first 10 serine residues from the N-terminal by alanine results in enhanced biological activity of the transgenic phytochrome, i.e., greater inhibition of hypocotyl growth in Rc or FRc. These observations suggest that the N-terminal may be involved in phytochrome signaling and that the serine residues may help in downregulation of the phytochrome signal.

Reciprocal domain swap experiments, in which chimeric constructs with the N-terminal domain of phyA fused to the C-terminal domain of phyB, or *vice versa*, are expressed transgenically, indicate that the N-terminal phytochrome with the chromophore determines the specificity of the signal, i.e., whether the chimeric protein behaves as phyA or phyB (Fig. 26-28). However, because the C-terminal domain is functional with the other N-terminal domain, it means that the transfer of signaling information from the photoreceptor to the recipient molecule involves the same biochemical mechanism. As shown in the following section, this mechanism could be a phosphorylation/dephosphorylation reaction.

1.2. Phytochrome as a Kinase

The concept that phytochrome may act as a light-activated enzyme and transfer its signal to a recipient molecule via phosphorylation is not new. In recent years, J. Clark Lagarias and associates at University of California, Davis, have shown that the phytochrome molecule is capable of acting as a kinase. In the mid 1980s, Lagarias and associates showed that purified phytochrome preparations from oat seedlings, in either Pr or Pfr form, were able to accept phosphate from radiolabeled ATP (autophosphorylation) and also transfer it to histone H1 or other proteins (i.e., act as a kinase); moreover, these activities were promoted or inhibited in a manner typical for Ser/Thr/Tyr-type protein kinases. For several reasons, however, the idea that

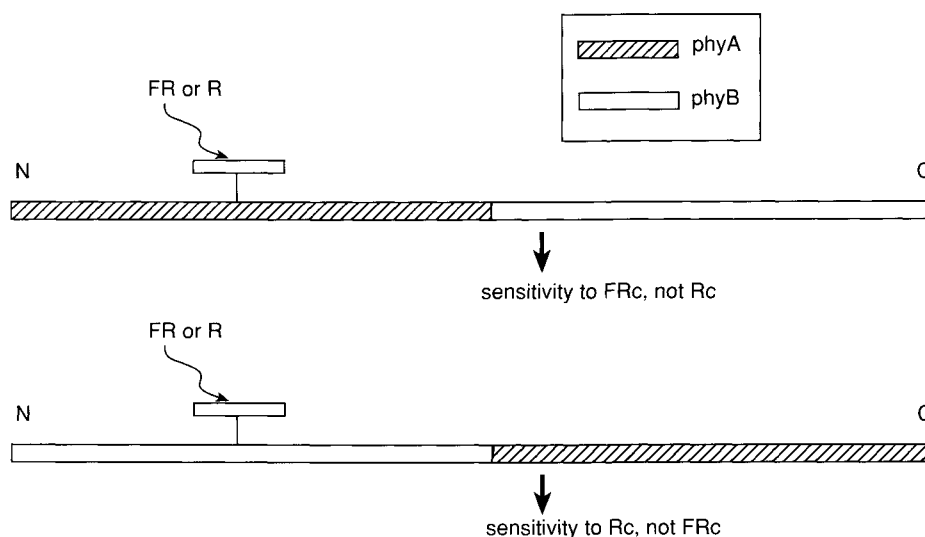


FIGURE 26-28 Chimeric constructs of phyA and phyB expressed in transgenic plants. The N-terminal half determines whether the chimeric photoreceptor behaves as phyA, i.e., the transgenic plant shows sensitivity to FRc, but not to Rc; or whether it behaves as phyB, i.e., shows sensitivity to Rc, but not to FRc.

phytochrome acted as a kinase was not seriously entertained. Some of the canonical domains of protein kinases, such as the ATP-binding motif of the Ser/Thr/Tyr protein kinase superfamily, and especially the catalytic domain typical of protein kinases (see Fig. 25-9 in Chapter 25) were absent from phytochrome sequences. There was also the possibility that the kinase activity seen in phytochrome preparations was due to a contaminant that copurified with the phytochrome.

1.2.1. Demonstration That Phytochrome Acts as a Kinase

The possibility that phytochrome may be a kinase was revived in 1991 when it was noted that the carboxy terminus of higher plant phytochromes had significant similarity to the histidine kinase domain of the sensor protein of prokaryotic two-component signaling systems (see Fig. 26-27). The idea was reinforced by the subsequent discovery of gene sequences in two cyanobacteria, which showed relatedness on the one hand to higher plant phytochromes and on the other to the histidine kinase domain of the bacterial sensor proteins.

The cyanobacterium *Fremyella diplosiphon* has a sensor kinase, which enables it to adapt to different light conditions. The N-terminal of this kinase shows a limited but significant sequence similarity to the N-terminal chromophore-bearing domain of higher plant phytochromes. *Synechocystis* is another cyanobacterium that has a phytochrome, now called Cph1 (for cyanobacterium phytochrome 1). The N terminus of Cph1 shows 30–35% amino acid identity to the chromophore-bearing domain of higher plant phytochromes. In addition, the C terminus of Cph1 contains the consensus sequences that define prokaryotic histidine kinases. Moreover, the operon that contains the open reading frame (ORF) for *CPH1* also contains an adjacent ORF that encodes a protein, now called Rcp1 (for response regulator for cyanobacterial phytochrome 1), which contains the conserved aspartate typical of response regulator proteins in bacterial two-component systems.

In a series of elegant experiments, Lagarias and co-workers showed first that the *Synechocystis* phytochrome acted as a typical prokaryotic two-component signaling system, where the light signal is transmitted *via* phosphate group transfer (Fig. 26-29A). Subsequently, they showed that oat phyA expressed in yeast was not only able to autophosphorylate itself, but also could transfer the phosphate group to a recipient protein (Fig. 26-29B). Significantly, however, phyA did not use the conserved histidine and aspartate for phosphotransfer, as in a typical prokaryotic system; instead it used serine for both autophosphorylation and transfer to the recipient protein. Both autophosphorylation and phosphotransfer to recipient protein showed greater enhancement by red light, i.e., in the Pfr form, than in the dark.

Thus, the higher plant phytochromes act as a kinase, but with a novel twist. They are Ser/Thr kinases without the canonical ATP-binding site or the catalytic domain of protein kinases. Several other kinases are now known that catalyze the ATP-dependent phosphorylation of their substrates at Ser residues, and similarly lack the conserved sequences of the eukaryotic Ser/Thr/Tyr protein kinase superfamily (e.g., mitochondrial protein kinases).

A model for higher plant phytochrome acting as a kinase in transfer of light signal is shown in Fig. 26-30A. It must be emphasized that this model is tentative because although some possible substrates for phosphotransfer have been identified (see below), their position and roles in phytochrome signaling are unknown. Also, mutants specific for phyA or phyB signaling (see Section III, 1.4) indicate the presence of other downstream signaling components.

The serine-rich domain at the N-terminal of higher plant phytochromes is lacking in the *Synechocystis* phytochrome Cph1 (Fig. 26-30B). Also lacking is the PAS-related domain (PRD). The PRD and the histidine kinase-like domain show considerable sequence similarity, and it has been hypothesized that the two sites arose by a duplication of an ancestral histidine kinase domain of the prokaryotic phytochrome. The specific roles of PRD and HKLD regions of eukaryotic phytochromes are still speculative. The PRD region with its PAS domain may be involved in phosphotransferase activity, as well as interaction with other proteins, whereas the HKLD region may serve to position a substrate. Recently, mutations specific to the histidine kinase domain of phyB were created. They indicate that the domain is important in phyB signaling; however, a mutation that is completely lacking in the histidine kinase domain still showed partial activity, suggesting that the domain is dispensable.

1.2.2. Substrates for Phytochrome Kinase Activity

In a search for possible substrates for phytochrome kinase activity, the carboxy terminus of phyA, considered important for signaling (see Section III, 1.1), was used as a bait in the yeast two-hybrid screen to fish out proteins that interacted with phytochromes (for this technique, see Appendix 1). Two proteins, phytochrome kinase substrate 1 (PKS1) and nucleoside diphosphate protein kinase 2 (NDPK2, but not NDPK1), from *Arabidopsis* have been identified that interact with phyA as well as phyB. PKS1 is phosphorylated in a light-dependent manner *in vitro*,

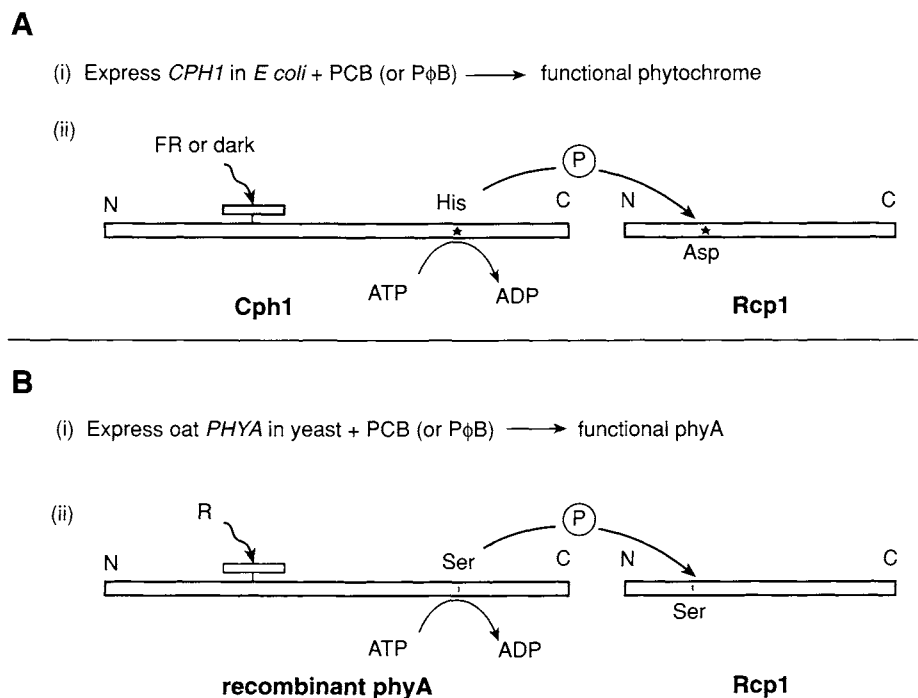


FIGURE 26-29 (A) (i) The *CPH1* sequence from *Synechocystis*, expressed in *E. coli*, gives a recombinant protein that binds to phycocyanobilin (PCB) or phytochromobilin (P Φ B) chromophores to yield a functional phytochrome with red/far-red photoreversibility. (ii) The recombinant Cph1-supplied radiolabeled ATP is autophosphorylated on the conserved histidine, which, in turn, transfers the phosphate to aspartate on Rcp1. Autophosphorylation and phosphotransfer to Rcp1 are mediated by FR light or in the dark, in the Pr form, unlike higher plant phytochromes. (B) (i) Oat *phyA* expressed in yeast and supplied PCB or P Φ B yields a functional phytochrome. (ii) The recombinant protein, supplied radiolabeled ATP, is able to autophosphorylate and to transfer the labeled phosphate group to Rcp1 from *Synechocystis*. Unlike *Synechocystis*, however, the radiolabeled amino acid residues, in the recombinant protein and in Rcp1, are not histidine and aspartate, respectively, but in both cases are serine; moreover, autophosphorylation is much more enhanced in R light (i.e., in Pfr form) than in the dark.

and its phosphorylation *in vivo* seems to be stimulated by red light. The PKS1::GFP fusion protein is localized in the cytoplasm, which indicates that it is a cytoplasmic protein. Since plants overexpressing PKS1 have longer hypocotyls in red light, PKS1 is thought to be a negative regulator of phyB signaling.

Nucleoside diphosphate kinases are multifunctional enzymes implicated in many developmental responses in *Drosophila*, rodents, and humans, although their role(s) in plants is unknown. They catalyze γ -phosphate group transfer and seem to act both as substrates for phosphorylation (using ATP and other nucleoside triphosphates) and as kinases. In etiolated pea epicotyls, an NDPK2 has been shown to be phosphorylated *in vivo* in light in a R/FR-reversible manner. The recombinant NDPK2 expressed in yeast shows enhanced phosphate transfer activity in a red light-dependent manner *in vitro*. These observations suggest that NDPK2 could be a substrate for phytochrome kinase activity. NDPK2-GFP constructs local-

ize the protein in both the cytoplasm and the nucleus. Loss-of-function *ndpk2* mutants show altered responses in both red and far-red light, which suggests that the kinase interacts with both phyA and phyB; but the *ndpk2* mutants are affected only in a subset of de-etiolation responses, i.e., greening of cotyledons and opening of the hook, not in hypocotyl elongation. Thus, NDPK2 probably acts at a branch point in the de-etiolation response. NDPK2 also appears to be a positive regulator of phyA and phyB signaling.

In summary, the higher plant phytochrome has been shown to act as a kinase, and at least two putative substrates for kinase activity have been identified, but whether phytochrome signaling involves a phosphorelay still remains to be shown. Meanwhile, data are accumulating that activated photoreceptors are translocated to the nucleus; also other intermediates in signaling are being identified using both the yeast two-hybrid screen and mutational analysis. These topics are covered next.

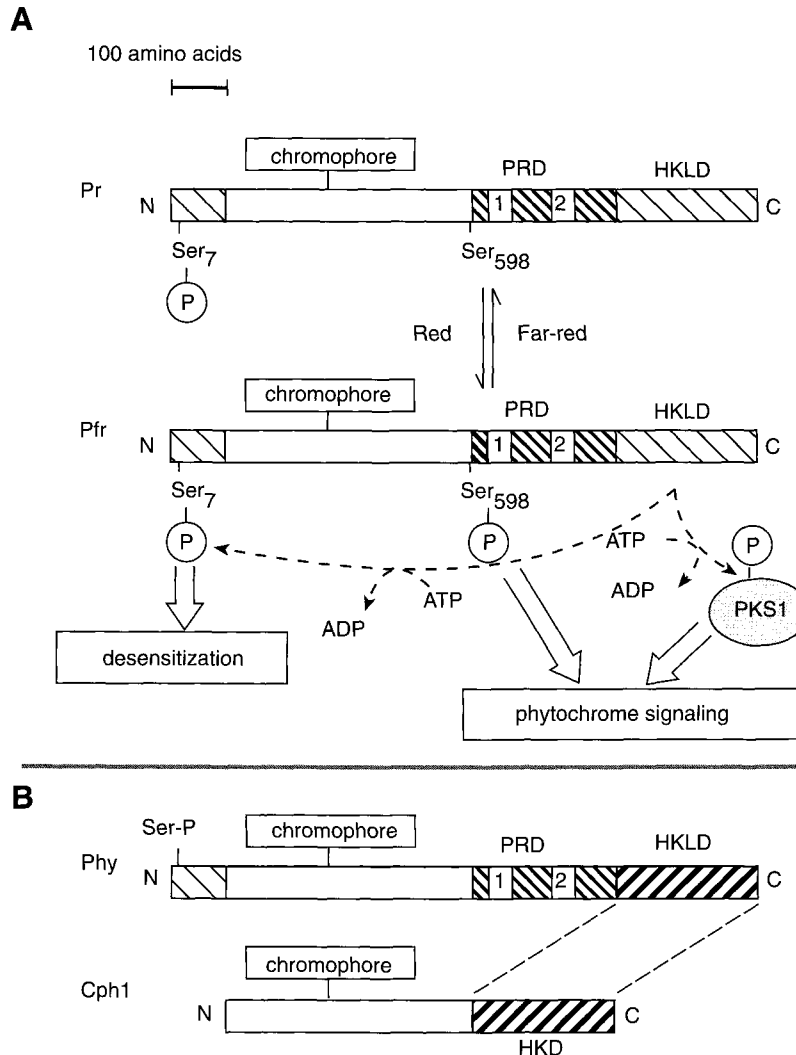


FIGURE 26-30 A model for higher plant phytochrome signaling and comparison of oat phyA and *Synechocystis* Cph1. (A) The model shows that red light stimulates phytochrome autophosphorylation at a serine residue (Ser₅₉₈) and transfer of a phosphate group to some substrate(s). A candidate substrate PKS1 is shown. Also, it is possible that a phospho-specific interaction occurs with a downstream element in the signaling cascade. Phosphorylation of the serine-rich amino-terminal region of phytochrome (Ser₇) is thought to downregulate the response. (B) Cph1 is a smaller protein and lacks the serine-rich domain at the N-terminal and the PAS-related domain in the C-terminal half of higher plant phytochromes. Adapted from Fankhauser and Chory (1999) and Elich and Chory (1997) with permission from Elsevier Science.

1.3. Activated PhyA and PhyB Are Localized to the Nucleus

Phytochromes have been traditionally thought to be soluble proteins present in the cytoplasm. In recent years, evidence has accumulated that phyA and phyB are translocated to the nucleus when activated by their respective irradiating wavelengths. Evidence comes from overexpression of a construct encoding a phytochrome and the green fluorescent protein (PHYA::GFP or PHYB::GFP) under a construct-

ive promoter, and/or transgenic expression of the construct in a plant such as tobacco. GFP does not disrupt normal plant responses, and the fusion protein can be localized intracellularly when activated by FRC or Rc (Fig. 26-31). Immunocytochemical staining with a phytochrome-specific (i.e., PHYA or PHYB) fluorescent antibody has been used for many years to localize individual phytochromes intracellularly. This method has been used in combination with a *phyA* null mutant as a control to provide greater specificity for phyA localization.

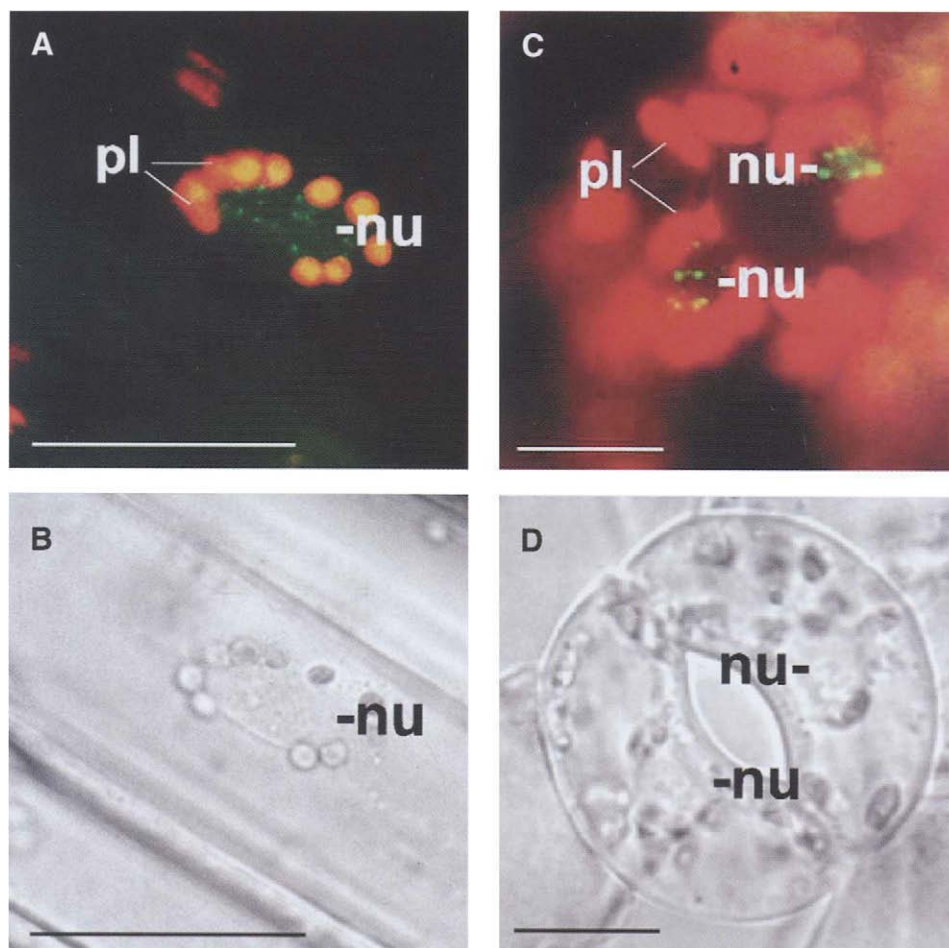


FIGURE 26-31 Nuclear localization of phyA-GFP and phyB-GFP in different cell types of light-grown transgenic tobacco plants. A and C are epifluorescence images, B and D are light microscope images. (A and B) Epidermal cell of a phyA-GFP-expressing tobacco seedling. (C and D) Stomatal guard cell from a 14-day-old phyB-GFP-expressing tobacco seedling. Pl, plastid; nu, nucleus. Scale bar = 20 μ m. From Kircher *et al.* (1999).

These studies have revealed that phyB in the PfrB form is translocated to the nucleus within minutes of irradiation by red light (Rc). If red light is given as a pulse, the translocation to nucleus is FR reversible. PhyA is also translocated to the nucleus, but there are some conflicting data. According to one report, phyA is translocated only under FR light. According to another report, translocation occurs under both R and FR light, but the kinetics of translocation of PfrA are much slower than those for PfrB; also, most of PfrA formed is degraded such that after a few hours it is undetectable. In contrast, PrA is much more stable. The implications of nuclear translocation on phytochrome signaling are still unclear.

The intracellular location of cry1 and cry2 has also been studied. Whereas, cry1 translocates to the nucleus in response to light, cry2 seems to be constitutively nuclear localized.

1.4. Intermediates in Phytochrome Signaling

Since different photoreceptors bring about some unique as well as some common photomorphogenic responses, it is reasonable to assume that each photoreceptor has unique signaling intermediates, as well as some common intermediates that it shares with other photoreceptors. To elucidate these intermediates in signaling, two approaches have been taken. One approach, as mentioned earlier, involves the use of the C-terminal domain of phytochromes as a bait to isolate proteins that interact with it in a yeast two-hybrid screen. Using this approach, an *Arabidopsis* cDNA clone was identified whose encoded protein, called phytochrome interacting factor 3 (PIF3), was shown to interact with both phyA and phyB.

PIF3 is a transcription factor with a β helix-loop-helix (bHLH) DNA-binding domain and is local-

ized to the nucleus in both light and dark. It also has a PAS domain. Thus, the protein has the structural competence to interact with DNA as well as other proteins. PIF3 is also biologically significant. Expressed transgenically in an antisense orientation, it reduced the inhibition of hypocotyl growth under Rc and, at lower fluence rates, under FRc (Fig. 26-32). Moreover, a T-DNA insertion mutant of *PIF3*, a dominant mutation called *poc1* (for photocurrent 1), shows enhanced sensitivity to red light in hypocotyl growth inhibition. On the basis of expression of PIF3 in an antisense construct and *poc1*, PIF3 is thought to be a positive regulator of phyB signaling.

The other approach is to isolate genetic mutants that are specific to a photoreceptor. Several class 3 mutants (see Fig. 26-14) have been identified in *Arabidopsis*. Some of them define steps that are common to

signaling by several photoreceptors (e.g., *hy5*), some define steps that are shared by phyA and phyB, and others seem to be unique to a specific photoreceptor (e.g., *hy1*, *red1*) (see Table 26-6). The wild-type genes for some of these mutants have been cloned (e.g., *HY5*, *SPA1*, *FAR1*; see also Table 26-7), but others have either not been cloned or are still not well characterized. However, judging from the effects of mutations on specific responses, it can be said that the signaling pathways for phyA, phyB, and cry1 are separate and branched, but they also converge at some loci.

Mutants that are selectively defective in phyA signaling show the phenotype of *phyA* synthesis mutants, i.e., seedlings show a long hypocotyl under continuous FR, but not under R, W, or B light. Similarly, mutants specific for phyB signaling have a phenotype of *phyB* mutants when grown under Rc light but not when grown under FRc, B, or UV-A light. Mutants for *cry1* and *cry2* have been isolated as well (*cry1*, *cry2*). Several mutants identified to date as phyA or phyB signaling mutants are not pleiotropically defective in all phyA- or phyB-mediated responses. Rather they represent later steps in signaling by phyA or phyB. For example, *hy1* is defective in only a subset of phyA-mediated responses (Fig. 26-33). The FR-mediated induction of *CAB* and *NITRATE REDUCTASE* (*NR*) genes is relatively unaffected in this mutant, but

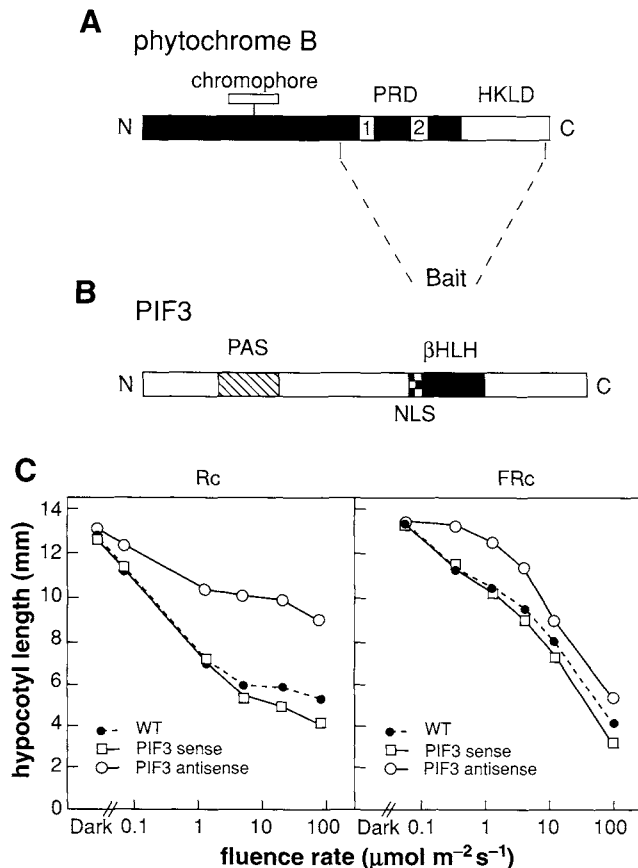


FIGURE 26-32 Isolation, structure, and activity of phytochrome-interacting factor 3 (PIF3). (A) A schematic for the bait used in the isolation of PIF3 in the yeast two-hybrid screen. (B) Structure of PIF3. A PAS domain, a nuclear localization signal (NLS), and β helix-loop-helix (β HLH) region are shown. (C) Inhibition of hypocotyl elongation in the wild-type and *Arabidopsis* plants transformed with *PIF3* cDNA in a sense and antisense orientation under continuous red (Rc) and far-red (FR) light. From Ni *et al.* (1998) with permission from Elsevier Science.

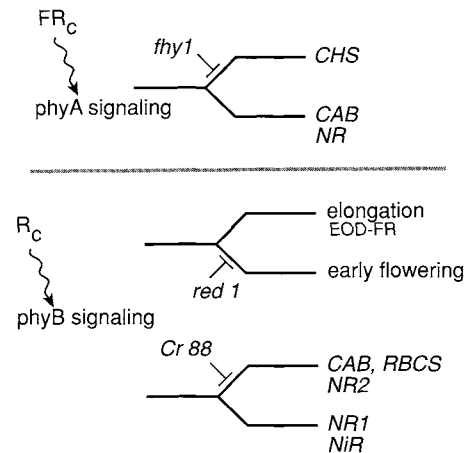


FIGURE 26-33 Diagrams illustrating that signaling pathways for phyA or phyB are branched. The *hy1* mutant, specific for phyA signaling, is defective in the FR-mediated induction of the *CHS* gene and anthocyanin synthesis, but is relatively unaffected in the induction of *CAB* and *NITRATE REDUCTASE* (*NR*) genes. The *red1* mutant, specific for phyB signaling, grown in light shows reduced inhibition of stem elongation and responses to EOD FR, but does not show early flowering. The *cr88* mutant, believed to be a phyB signaling mutant, is impaired in the light-induced expression of *NR2*, *CAB*, and *RBCS* genes, but shows wild-type induction of *NR1* and *NITRITE REDUCTASE* (*NiR*) genes.

TABLE 26-6 Representative *Arabidopsis* Mutants in Photoreceptor Signaling

Signaling pathway	Mutant	Screen	Ref.
phyA	<i>fhy1, fhy3</i> for far-red long hypocotyl	Long hypocotyl under FRc	Whitelam <i>et al.</i> (1993); Barnes <i>et al.</i> (1996)
	<i>spa1</i>	Extragenic suppression of a weak <i>phyA</i> mutation	Hoecker <i>et al.</i> (1998)
	<i>fin2</i> (for far-red insensitive)	Insensitivity to FRc, but not to Rc	Soh <i>et al.</i> (1998)
	<i>far1</i> (for far-red impaired response)	Extragenic suppression of <i>phyA</i> overexpression	Hudson <i>et al.</i> (1999)
phyB	<i>pef2, pef3</i> (for phytochrome early flowering)	Early flowering under LD and SD	Ahmad and Cashmore (1996)
	<i>red1</i>	Extragenic suppression of <i>phyB</i> overexpression	Wagner <i>et al.</i> (1997)
phyB	<i>cr88</i> (for chlorate resistance)	Resistance to chlorate	Lin and Cheng (1997);
both phyA and phyB	<i>pef1</i>		Ahmad and Cashmore (1996)
phytochromes and B/UV-A photoreceptors	<i>hy5</i> (for long hypocotyl)	Long hypocotyls under R, FR, B, UV-A	Koornneef <i>et al.</i> (1980); Oyama <i>et al.</i> (1997)

induction of the *CHS* gene and anthocyanin synthesis under FR are deficient. This means that *FHY1* is involved in the regulation of *CHS* gene expression, but not in the expression of *CAB* or *NR* genes. These data, while confirming that the induction of *CHS* and *CAB* genes represents distinct branches of the *phyA* signaling pathway, also agree with the observation that *CHS* is expressed via phytochromes and B/UV-A only in the seedling; in mature leaves, it is induced exclusively via B/UV-A photoreceptors. In addition, another study indicates that *fhy1* mutants are impaired in high irradiance responses, but not in VLF responses, which suggests different modes of signaling for VLF and HI responses mediated by *phyA*.

A mutant specific to *phyA* signaling has been obtained using an elegant screening protocol. As explained in Section II earlier in this chapter, seedling de-etiolation under FRc is mediated via *phyA*. Wild-type seedlings grown in dark and given FRc stop elongating, the apical hook opens, and cotyledons unfold. However, FRc does not activate protochlorophyllide oxidoreductase; hence, protochlorophyllide formed in etioplasts in the dark is not converted to chlorophyll and the plants do not turn green, even though the prolamellar bodies typical of etioplasts, which normally give rise to thylakoids on exposure to white light, disaggregate and vesicles disperse in the stroma. These etioplasts with disaggregated prolamellar bodies are unable to convert to chloroplasts subsequently on exposure to white light, with the result that the seed-

lings eventually die—a FR-induced death. Typical *phyA* mutants, such as *fhy1*, do not respond to FRc and retain long hypocotyls, a modest hook, and folded cotyledons. Moreover, on subsequent exposure to white light, they green and do not die. This combination of observations provided a screening strategy to select mutants specific to *phyA* signaling. A population of T-DNA-tagged mutagenized *Arabidopsis* seedlings was kept in FRc for 84 h and then in white light for 3 days and screened for greening. Surviving plants with longer hypocotyls than the wild type were grown to maturity, and the progeny was rescreened under both FR and red light. Only plants with long hypocotyls under FR but not under red light conditions were specifically impaired in *phyA* signaling.

The mutant identified, *pat1* (for, phytochrome A signal transduction), is impaired in most *phyA*-mediated responses, including de-etiolation under FRc and *CAB* and *CHS* gene induction. Thus, it is thought to play a role early in *phyA* signaling. The wild-type gene *PAT1* encodes a new member of the GRAS family of transcription factors (see Table 26-7). As mentioned in Chapter 24, GA signaling and GAI/RGA-type proteins in *Arabidopsis* and their orthologs in wheat and maize, along with SCARECROW (*SCR*) in *Arabidopsis*, represent a novel class of transcription factors with diverse functions, which so far have been found only in plants. *PAT1* shares little sequence homology with GAI/RGA or *SCR*, but it shares the domain structure with these proteins and has the canonical two leucine

TABLE 26-7 Molecularly Characterized Intermediates in Phytochrome Signaling

Intermediate	Specific action	Characteristics (cellular location)	Ref.
PIF3	A positive regulator of phyB	bHLH transcription factor (nucleus)	Halliday <i>et al.</i> (1999)
PKS1	Negatively regulates phyB	Substrate for phytochrome kinase activity (cytoplasm)	Fankhauser <i>et al.</i> (1999)
NDPK2	Positive regulator of phyA and phyB in cotyledon opening and hook straightening	Substrate for phytochrome kinase activity (cytoplasm and nucleus)	Choi <i>et al.</i> (1999)
FAR1	Positively regulates phyA signaling	Protein with a coiled-coil domain (nucleus)	Hudson <i>et al.</i> (1999)
SPA1	Negative regulator of phyA	WD repeat protein (nucleus)	Hoecker <i>et al.</i> (1999)
PAT1	Positive regulator of phyA signaling	GRAS-type transcription factor (cytoplasm)	Bolle <i>et al.</i> (2000)
HY5	Downstream regulator for phyA, phyB, and cry1	bZIP transcription factor (nucleus)	Oyama <i>et al.</i> (1997)

heptad repeat sequences with an intervening VHIID-type domain (see Fig. 24-13 in Chapter 24). However, unlike GAI/RGA or SCR, it lacks any nuclear localization sequence, and the PAT1::GFP fusion protein is localized predominantly in the cytoplasm.

The *hy5* mutant, isolated in the initial screen for long hypocotyls, shows elongated hypocotyls under continuous R, FR, B, and UV-A, and, hence, identifies a common step in the signaling pathways for phytochromes as well as cryptochromes. It is defective in light-induced chlorophyll and anthocyanin accumulation and shows extensive root abnormalities. Genetic analysis indicates that HY5 acts downstream from the convergence of signaling pathways of phytochromes and cryptochromes. The *HY5* gene encodes a bZIP-type transcription factor (see Table 26-7) that is localized in the nucleus and has been shown to bind to the ACGT core containing G-box elements of light responsive genes.

Many other signaling intermediates, in addition to those listed in Table 26-6, have been identified in the last year. Some are specific to phyA signaling (e.g., EID1 for *embfindlicher im dunkelroten Licht* 1), some to phyB signaling (e.g., ELF3, for early flowering 3), some that are common to phyA and phyB (e.g., PKS2), and some that act further downstream and participate in signaling by multiple photoreceptors.

In summary, many intermediates in phytochrome signaling have been identified by the yeast two-hybrid screen or mutational analysis, and many more are being identified each year. The positions at which these intermediates act in signal transduction, or the upstream and downstream elements that they interact with, are still largely a matter of conjecture, but rapid

progress is being made. Among these intermediates, some act at earlier steps, others later in signaling. HY5, which acts downstream of the convergence of phytochrome and cryptochrome signaling most likely acts at or near the final steps of signal transduction. Phytochrome and cryptochrome signaling is also subject to negative regulation by a group of proteins. That topic is covered next.

1.5. Negative Regulators

A series of mutants in *Arabidopsis* have been isolated in screens for a de-etiolated phenotype in dark-grown seedlings (class 4 in Fig. 26-14). These mutants, isolated in different laboratories and named as de-etiolated (*det*) or constitutively photomorphogenic (*cop*), have similar phenotypes to a group of mutants known as *fusca* (*fus*), which were identified through a screen for purple seed color. *fus* Mutants accumulate large amounts of anthocyanins in embryo and seedlings and usually are lethal. Many *DET* and *COP* loci turned out to be identical to *FUSCA* loci, and, hence, they are all collectively referred to as *COP/DET/FUS* loci. At least 16 such loci are known, with many mutant alleles at each locus. Eleven loci are pleiotropic in their effects and regulate a large number of responses associated with de-etiolation; others are restricted to specific cell or tissue types. Since each is a recessive mutation, it means that the WT genes encode proteins that act to repress de-etiolation in the dark or, in other words, maintain the etiolated state in dark-grown seedling. When mutated, the de-etiolated phenotype is released; thus, they act as negative regulators of seedling photomorphogenesis

(Fig. 26-34). As discussed in Section IV, several hormones interact with light signaling, and some mutants initially identified as *det/cop* mutants have turned out to be mutations in brassinosteroid biosynthesis.

COP/DET/FUS loci are epistatic to each of the known photoreceptor mutations (e.g., *hy1*, *hy2*, *phyA*, *phyB*, *cry1*), indicating that they act downstream from photoperception by phytochromes or the cryptochrome, *cry1*. They are also downstream of many, not all, signaling mutants listed in Table 26-6. Thus, proteins encoded by *COP/DET/FUS* loci act downstream of convergence of phytochrome and cryptochrome signaling pathways.

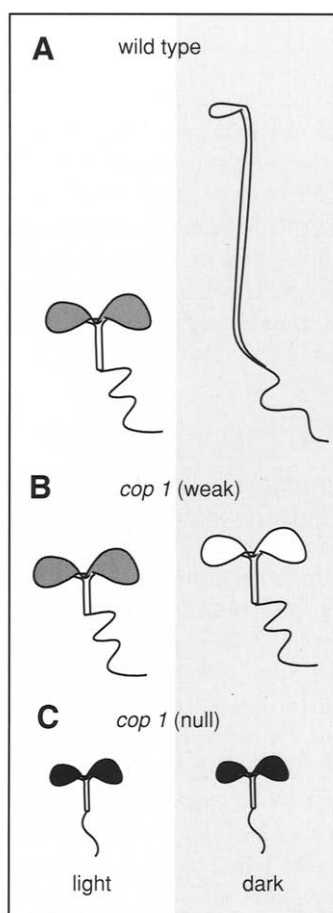


FIGURE 26-34 Representations of *Arabidopsis* wild-type and *cop1* mutant seedlings grown in light or in darkness. (A) Wild-type seedlings show a de-etiolated phenotype in light and an etiolated phenotype in darkness. (B) A weak allele of *cop1*. Light-grown seedlings of *cop1* exhibit hypersensitivity to light, and hypocotyl elongation is even further inhibited than in the wild type; dark-grown seedlings display photomorphogenesis. (C) A null allele of *cop1*. Seedlings displaying constitutive photomorphogenesis accumulate very high levels of anthocyanin (*fusca* phenotype) and are lethal. From Torii and Deng (1997) with permission.

1.5.1. Negative Regulators Repress the Transcription of Light-Regulated Genes

The various mutants at the pleiotropic *DET/COP/FUS* loci show essentially identical phenotypes (see Fig. 26-34 for *cop1*), which suggests that the WT genes encode proteins that function in close association with each other or alternatively form subunits of a multiprotein complex. Many *DET/COP/FUS* genes have been cloned. Their mRNAs are constitutively present in all cell and tissue types and are expressed independently of light. One gene, *COP1*, which encodes an unusual protein, plays a central role in the repression of seedling photomorphogenesis in dark, and its overexpression in transgenic plants inhibits normal photomorphogenesis in light.

1.5.1.1. *COP1* Protein

The *COP1* protein is predicted to have some well-recognized motifs (Fig. 26-35). It has a zinc-binding domain, which could be involved in binding to DNA, and two other motifs, a coiled-coil helix and WD40 repeats, which could be involved in protein-protein interactions. *COP1* also shows significant and extended sequence similarity to one of the proteins in the *Drosophila* transcription factor (TFIID) complex, part of the general transcription machinery involving RNA polymerase II. Thus, *COP1* has the structural basis for interacting with both DNA and proteins and could repress gene expression.

COP1::GUS fusion constructs expressed in transgenic plants indicate that *COP1* is located predominantly in the nucleus in darkness, but in light it is translocated to the cytoplasm. This observation is supported by its distribution in plant parts. In above-ground parts, which show de-etiolation, most of the concentration of *COP1* is localized in the nucleus in darkness, but in light there is a greater concentration in the cytoplasm. In root tissue, as expected, *COP1* occurs strictly in the nucleus because roots are not normally exposed to direct light or turn green.

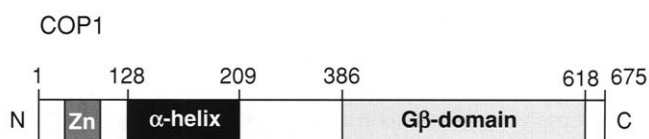


FIGURE 26-35 Structure of *COP1*. The protein has a ring finger Zn-binding domain at the N-terminal (Zn), a coiled coil helix (α -helix), and several WD-40 repeats, typical of the β -subunit of heterotrimeric G-proteins ($G\beta$ -domain), at the C-terminal. The amino acids flanking the domains are numbered.

1.5.1.2. COP9 Signalosome

Several other *COP/DET/FUS* genes encode nuclear-localized proteins that occur as subunits of a large multimeric complex, known as the COP9 signalosome. The COP9 signalosome in both *Arabidopsis* and cauliflower has eight subunits, many of which have been characterized at the molecular level in *Arabidopsis*. The assembly and stability of the COP9 signalosome in the nucleus depend on the functional integrity of all the constituent polypeptides. In the event of a mutation in any of the genes encoding these polypeptides, the COP9 complex fails to assemble or be stable.

In the nucleus, the COP9 signalosome is thought to interact with other proteins, including DET1 and COP1. DET1 is localized in the nucleus, but the nuclear localization of COP1 depends not only on darkness, as mentioned earlier, but also on the functional integrity of the constituents of the COP9 signalosome. Although direct evidence is lacking, COP1 is believed to partner with the COP9 signalosome. Other possible partners include DET1 and COP10. The precise roles of these partners of COP1 in repressing the photomorphogenic program in the dark are unknown, but they may be involved in maintaining COP1 integrity in the nucleus.

1.5.1.3. Proposed Model

In vitro analysis indicates that COP1 interacts with HY5, a bZIP transcription factor localized in the nu-

cleus. As mentioned earlier, *hy5* mutants show a tall phenotype under R, FR, and B light; hence, HY5 is thought to be a positive regulator of seedling photomorphogenesis. HY5 has also been shown to bind to the promoter regions of some light responsive genes (e.g., *CAB* and *CHS*) in *in vitro* assays. It is proposed that COP1 dimers bind to HY5 and repress gene expression associated with photomorphogenesis (Fig. 26-36). CIP7 is another positive regulator of photomorphogenesis, and COP1 has been shown to interact with it as well. There may be other still unknown positive regulators of photomorphogenesis repressed by COP1.

Perception of the light signal by photoreceptors and its transduction results in depletion of the COP1 concentration in the nucleus and a release of the repression of the positive regulators of light-regulated genes. Since the COP9 signalosome, and possibly also COP10 and DET1, affects COP1 stability in the dark, light signals may also interfere with that association.

How the light signal is transduced to the nucleus is unknown. It may be transduced directly from the activated photoreceptors, several of which are now known to be translocated to the nucleus in an activated state (e.g., phyA, phyB, cry1). Alternatively, some signaling intermediates may be involved.

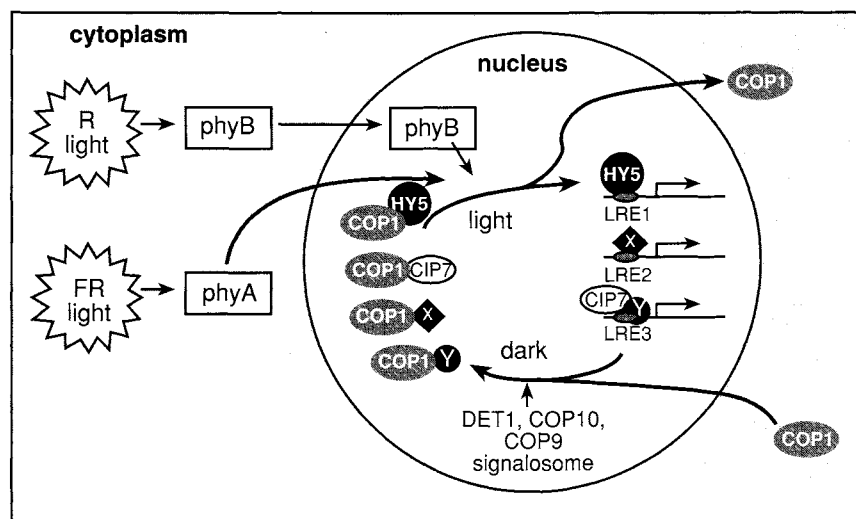


FIGURE 26-36 A proposed model for light signaling events leading to seedling photomorphogenesis. Only two photoreceptors, phyA and phyB, are shown. The light signals perceived by these photoreceptors are shown as being transduced by distinct pathways; they serve to modulate either COP1 itself and/or its partners, COP9, COP10, and DET1. As a result of this modulation, COP1 concentrations (or activity) are depleted in the nucleus, relieving the repression of transcription factors, such as HY5, CIP7, and still unknown factors X and Y. Once relieved, these transcription factors positively regulate the expression of genes involved in seedling photomorphogenesis. LRE, light responsive element. From Deng and Quail (1999).

1.5.2. COP/DET/FUS Loci Have Other Roles

While the role of COP1 and COP9 signalosome in repression of the de-etiolation program is clear, there is growing evidence that these proteins have other roles as well. For example, the severe mutant alleles at *COP/DET/FUS* loci are embryo or seedling lethal, which suggests that their encoded proteins are involved in other processes in light besides de-etiolation. Weak mutant alleles lead to the inappropriate expression of many other genes, including some involved in pathogenesis or stress responses, and in developmental programs that are not regulated by light. It has also been found that homologues of COP9, FUS6/COP11, and COP8, which form subunits of the COP9 signalosome, occur in many other organisms, including humans, mice, and nematodes. Moreover, the COP9 signalosome is conserved between mammals and plants, and its subunits exhibit sequence similarity to non-ATPase subunits of the 19S regulatory particle of the 26S proteasome, the protease for ubiquitinated proteins (see Box 22-2 in Chapter 22). The significance of this similarity in functioning of the complex is not clear, but it has been suggested, that the COP1-HY5 interaction may specifically target HY5 for proteasome-mediated degradation in the nucleus.

1.6. Signaling via G-Proteins and Secondary Messengers

Some authors have suggested that phytochrome-induced gene expression proceeds via G-proteins and secondary messengers, such as calcium/calmodulin or cGMP (see Chapter 25). Calcium transients have been reported in tobacco protoplasts given a pulse of red light. More detailed work has been done using the *aurea* mutant of tomato, which produces a defective chromophore (see Fig. 26-8) and, hence, lacks normal phytochrome-mediated responses, such as chloroplast biogenesis and accumulation of anthocyanin.

If a purified extract of oat phyA in the Pfr form is injected into hypocotyl cells of the *aurea* mutant, a complete restoration of the photomorphogenic program is obtained. This includes conversion of etio-plasts into chloroplasts and expression of photosynthetic genes (e.g., *CAB*, *RBCS*), as well as expression of the *CHS* gene (encoding the enzyme chalcone synthase) and accumulation of anthocyanin in the vacuoles (Fig. 26-37). Injection of cells with GTP γ S or cholera toxin (CTx), which lock the heterotrimeric G-proteins in an activated state (see Box 25-1 in Chapter 25), also restores the morphogenic program in the absence of light, which indicates that phyA signaling progresses via heterotrimeric G-proteins. In further

work, it was shown that signaling via G-proteins branched into three pathways, one via calcium/calmodulin led to the expression of *CAB* genes, the other via cGMP led to expression of anthocyanin synthesis genes, such as *CHS*, and the third leading to complete chloroplast development and anthocyanin accumulation involved both. Subsequently, it was shown that the phyA-induced repression of genes, such as asparagine synthetase (*ASI*), also proceeds via heterotrimeric G-proteins.

These results are not easy to correlate with the known intracellular distribution of phyA, nor is it clear how G-proteins get activated. For G-protein signaling, a membrane-bound receptor is required, whereas phytochrome is a soluble photoreceptor, which occurs in the cytoplasm as well as in the nucleus.

1.7. From Signaling Components to Gene Expression

The promoter sequences of many light-induced genes have been analyzed for the identification of *cis* sequences that are important for light-induced expression, as well as transcription factors that bind to them. Conserved *cis* elements described as G box or E box are common in these promoters, and many different types of transcription factors (e.g., bZIP, Myb) have been shown to bind to them. Despite a wealth of information, however, no single conserved *cis* element is found in all light responsive promoters, and the smallest promoter sequences found to confer light inducibility on heterologous minimal promoters are multipartite elements that contain different combinations of *cis*-acting sequences. Moreover, the response complexes so identified still retain the dependence of light wavelength, developmental state of chloroplasts, and tissue specificity of the native promoter. Finally, although numerous proteins have been shown to bind to these sequences, none has been shown to uniquely regulate all light-induced genes. It appears, therefore, that different combinations of *cis* sequences and transcription factors regulate specific light-induced genes within a developmental context.

Among the molecularly characterized intermediates in phytochrome signaling (see Table 26-7), some are transcription factors located in the nucleus. Among these, HY5 is best characterized and, as mentioned earlier, positively regulates light responsive genes and is itself negatively regulated by COP1. *In vitro* binding assays have shown that PIF3, although it is a bHLH protein, binds to the G-box sequence in promoters of light-regulated genes, such as those associated with photosynthesis and the circadian clock. Furthermore,

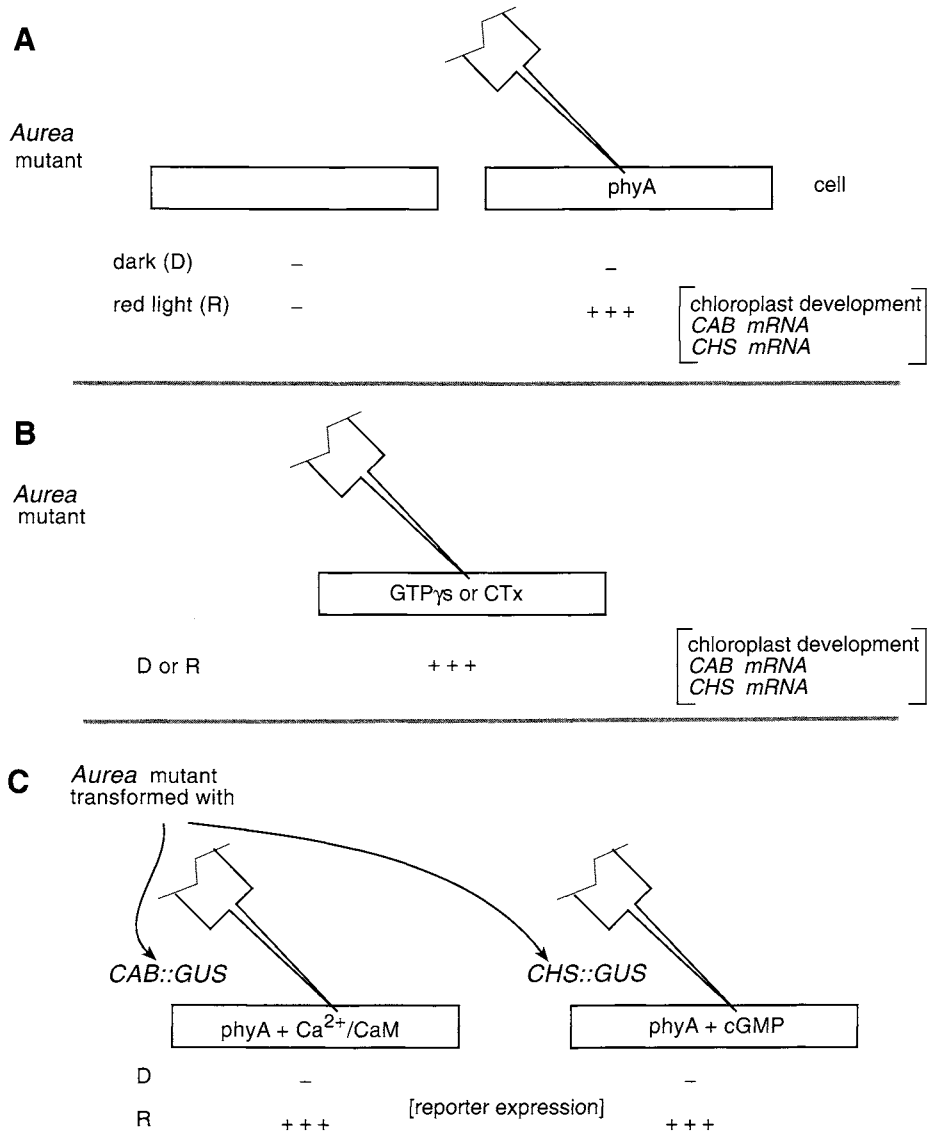


FIGURE 26-37 Schematic representation of phyA signaling using the *aurea* mutant of tomato. (A) Epidermal cells were injected with +/- oat phyA under either white or red light to produce activated PfrA or in darkness to give PrA. Cells injected with phyA in the Pfr form showed complete development of chloroplasts, including expression of CAB mRNA, as well as anthocyanin accumulation, whereas those injected with PrA did not. (B) Cells of the *aurea* mutant injected with GTP γ S or cholera toxin show light-independent development of chloroplasts and anthocyanin accumulation. (C) *aurea* mutants were transformed with CAB::GUS or CHS::GUS cosntructs. Epidermal cells coinjected with PfrA and calcium/calmodulin show CAB::GUS expression, whereas those coinjected with PfrA plus cGMP show CHS::GUS expression. Adapted from data in Neuhaus *et al.* (1993).

the Pfr form of phyB (PfrB) is bound to PIF3 in the complex in a R/FR reversible manner. These data suggest that PfrB in the nucleus triggers transcription of light-responsive genes via PIF3 in one of the most direct and short signaling pathways, somewhat similar to that of steroid receptors in mammalian systems. PAT1 is cytoplasmic, Yet is thought to be involved in regulation of CAB or CHS gene expression in an unknown manner. PKS1 is also cytosolic. Since it nega-

tively regulates phyB action, it has been speculated that it does so by preventing phyB from being translocated into the nucleus. For others, no connecting links are known between phytochrome signaling and induction of light responsive genes. In an effort to known more about the range of genes up- or down-regulated by the light signal, the technique of DNA microarrays (see Appendix 1) is being used. Results from these studies will be awaited with interest.

1.8. A Model for Light Signaling

The information just summarized does not allow a coherent and consistent model for phytochrome and cryptochrome signaling. Still, on the basis of available data from epistasis studies on mutants and known intermediates in phytochrome A and B signaling, a tentative and simplified scheme is shown in Fig. 26-38. In this scheme, light signals perceived by individual photoreceptors are transduced through their specific signaling partners and then funneled into a common processor, which includes members of the COP/DET/FUS class of proteins. PhyA and phyB share a common pathway, which also funnels into the common processor. In the absence of light, the output of the common processor keeps the photomorphogenic program in check by inhibiting proteins such as HY5. In the presence of light, however, output from the processor is diminished and results in activation of the downstream transcription factors and gene expression. Since the effects of light signaling also include modulation of elongation growth, expansion of leaves, and stomatal movements, the downstream cascade also involves changes in the cytoskeleton and ion channels.

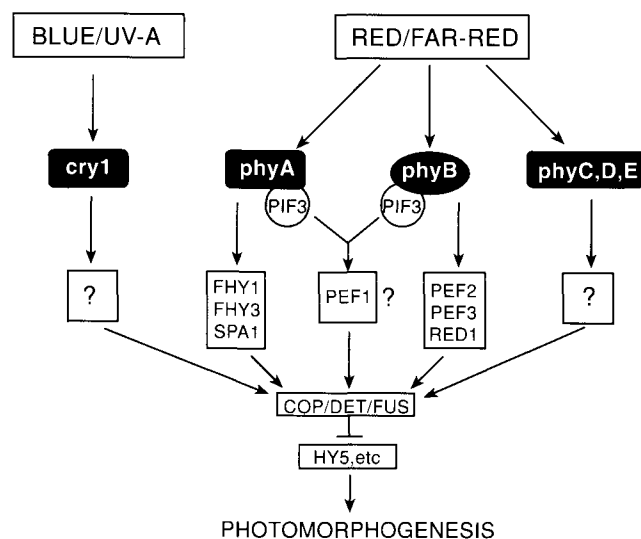


FIGURE 26-38 A simplified scheme illustrating proposed signaling pathways for phytochromes and cryptochromes. Potential signaling components specific to the phyA and phyB pathways are identified, but not for cry1, cry2, phyC, phyD, or phyE because they are unknown. Dual signaling pathways are proposed to emanate from phyA and phyB. A shared pathway via PIF3 interacts with both photoreceptors. Also, there are initially separate pathways, which are specific to phyA (via FHY1, FHY3, and SPA1) or to phyB (via PEF2, PEF3, and RED1). Some factors may function in the shared pathway (e.g., PEF1). The signaling pathways from various photoreceptors converge at, or upstream of, the COP/DET/FUS group of regulators, which negatively regulate HY5 and other downstream factors. Modified from Deng and Quail (1999).

The model does not account for the possible role of phytochrome A (and, by implication, other phytochromes) as protein kinases that may transfer the light signal via phosphate group transfer. The model also does not include G-proteins and secondary messengers, such as cGMP and Ca^{2+} /calmodulin. Their position in the model is uncertain. Many light-mediated responses are also mediated via hormones, and there is evidence for cross talk between elements of light signaling and elements of hormonal signaling. That part is also excluded from the model. That subject is covered in the next section.

2. SECTION SUMMARY

The manner in which the light signal from photoreceptors is transferred to the recipient molecules and the subsequent steps in signal transfer related to biological responses (e.g., gene expression, cytoskeletal reorganization, ion channel control) are still very much a mystery. Very little is known about signal transduction with respect to the cryptochromes. More is known about phytochromes, but although there are several promising leads, there is no scheme that links the various pieces of the puzzle into a coherent, meaningful picture. Two adjoining domains in the phytochrome molecule, the PAS-related domain and the histidine kinase-like domain in the C-terminal half, are implicated in phytochrome signaling. There are convincing data that phytochrome, on being activated by red light, is autophosphorylated at a serine residue and subsequently donates that phosphate group to a serine residue in a recipient molecule. This may well be the first step in signal transduction, but the substrate(s) for such a transfer is unknown. It has also been shown that phytochromes can act as kinases, and some putative substrates for kinase activity have been identified, but it remains to be shown that phytochrome signaling involves phosphate group transfer or transfer cascade.

Several factors that interact with phyA, phyB, or both have been isolated by mutant analysis or in yeast two-hybrid screens. The genes for several of them have been cloned. At least a few of them have been shown to participate in the phytochrome-mediated de-etiolation program, either positively or negatively. Some are localized primarily in the nucleus and others in the cytoplasm, but the details of their participation are unknown. The *Arabidopsis* HY5 gene encodes a transcription factor that positively regulates the seedling photomorphogenic program under a variety of light regimes. Hence, it represents a locus common to both phytochrome and cryptochrome signaling. Moreover, in one of the most direct signaling pathways, PfrB in complex with a

transcription factor, PIF3, has been shown to bind to the promoter sequences of some light-responsive genes. Several negative regulators of the de-etiolation program are known. One of them, COP1, occurs in the nucleus in the dark, but is partitioned into the cytoplasm in light and is thought to play a central role in the repression of light-regulated genes. COP1 is believed to interact with other negative regulators in the nucleus, specifically with a large multimeric complex, the COP9 signalosome. According to one model, COP1 represses photomorphogenesis by binding to HY5 and other positive regulators and thus inhibits photomorphogenic gene transcription. This repression is relieved on perception and transduction of the light signal by photoreceptors, which results in a depletion of COP1 concentration in the nucleus and its migration to the cytoplasm. The details of signal transduction to the nucleus, whether by activated photoreceptors or by some intermediates, are unknown. Microinjection of phyA and pharmacological studies have implicated heterotrimeric G-proteins as well as cGMP and Ca^{2+} / CaM in mediating phytochrome-induced responses associated with de-etiolation. Thus, while many individual pieces are known, there is as yet no clear picture as to how they fit with each other to affect phytochrome-mediated responses.

SECTION IV. PLANT HORMONES AND LIGHT SIGNALING

1. LIGHT AND HORMONES SHARE REGULATION OF MANY PLANT PROCESSES

Many processes that are regulated by light are also regulated by hormones. Three hormones in particular, gibberellins, indoleacetic acid (IAA), and cytokinins (CKs), but also brassinosteroids (BRs), and possibly abscisic acid (ABA), are involved in many of the same responses as are affected by light. The question arises whether the hormones and photoreceptors act independently or whether the multiple signaling pathways intersect? We begin by examining the interaction between phytochromes and gibberellins.

1.1. Gibberellin and Phytochrome Interactions

Responses such as germination of seeds, stem elongation, including bolting in rosette plants, de-etiolation of seedlings in light, and flowering are all regulated by light and GAs. These responses are very

different from one another and probably involve induction, or repression, of different isoforms of GA metabolism enzymes in a tissue-specific manner. Data providing a link between phytochromes and the transcription of genes encoding enzymes catalyzing synthesis or inactivation of active GAs are still very few. Such a link probably occurs at the level of GA 20-oxidase and/or GA 3 β -hydroxylase, enzymes that catalyze the two later steps in the synthesis of bioactive GAs (see Section 5.4, Chapter 7). It may also occur at the level of 2 β -hydroxylases, enzymes that inactivate GAs. The earlier steps in synthesis, i.e., cyclization of geranylgeranyl diphosphate to *ent*-kaurene (mediated by copalyl diphosphate synthase and *ent*-kaurene synthetase) and oxidation of *ent*-kaurene to *ent*-kaurenoic acid (by various P450 monooxygenase-linked oxidases), are not light regulated. For instance, the mutants *ga1 Arabidopsis* or *d5 maize*, which are blocked at copalyl diphosphate synthase and *ent*-kaurene synthetase, respectively, remain dwarf both in light and in dark. In contrast, *d1 maize* and *le pea*, both blocked at the 3 β -hydroxylase step, are dwarfs only in light, not in dark. Several GA 20-oxidase (GA 20-ox), GA 3 β -hydroxylase (GA3 β -hy), and GA 2 β -hydroxylase (GA2 β -hy) cDNAs and genes have been cloned (see Chapter 7). However, since these enzymes are encoded by multigene families (as opposed to earlier acting enzymes, which are encoded by single copy genes), in order to unequivocally show a phytochrome effect, it is essential to isolate a cDNA (or gene) that encodes an isoform that is specifically involved in GA synthesis (or inactivation) related to a particular response. So far this has been accomplished only with seed germination.

1.1.1. Germination of Photoblastic Seeds

We have seen that germination in photoblastic seeds is triggered by low fluence red light in a R/FR reversible manner, and this response is mediated primarily by phyB or type II phytochromes. It is also mediated by phyA acting in a VLF mode. As explained in Chapter 19, seed germination is also modulated by a high GA/ABA ratio. Moreover, in many cases (e.g., lettuce seeds of variety Grand Rapids), the red light requirement of seeds is almost completely bypassed by placing seeds in a GA solution in the dark (Fig. 26-39).

The replacement of red light requirement by exogenous GA raises the possibility that one of the actions of activated phytochrome is enhanced synthesis of biologically active GAs. Quantitative gas chromatography-mass spectroscopy has shown that endogenous GA₁ levels in lettuce seeds rise about three times that of dark control 8 h after red light irradiation, and that this effect is R/FR reversible (Fig. 26-39).

Two genes encoding 3β -hydroxylases have been cloned from lettuce. One of them is expressed predominantly in seed tissues and is induced by a short exposure to red light in a R/FR reversible manner. Analysis of the phytochrome involved was not possible in lettuce. Fortunately, two 3β -hydroxylase genes have also been cloned in *Arabidopsis* (*GA4* and *GA4H*). One of them (*GA4H*) is expressed predominantly in seeds

and young seedlings, and its expression is induced within 1 h by an exposure of wet seeds to red light and reversed by FR light (Fig. 26-40). Furthermore, it could be shown in *Arabidopsis* that phyB is the phytochrome involved because *GA4H* mRNA expression is missing in the *phyB* mutant. In contrast, the *GA4* gene is not regulated by red light and is also expressed in vegetative tissues of the adult plant.

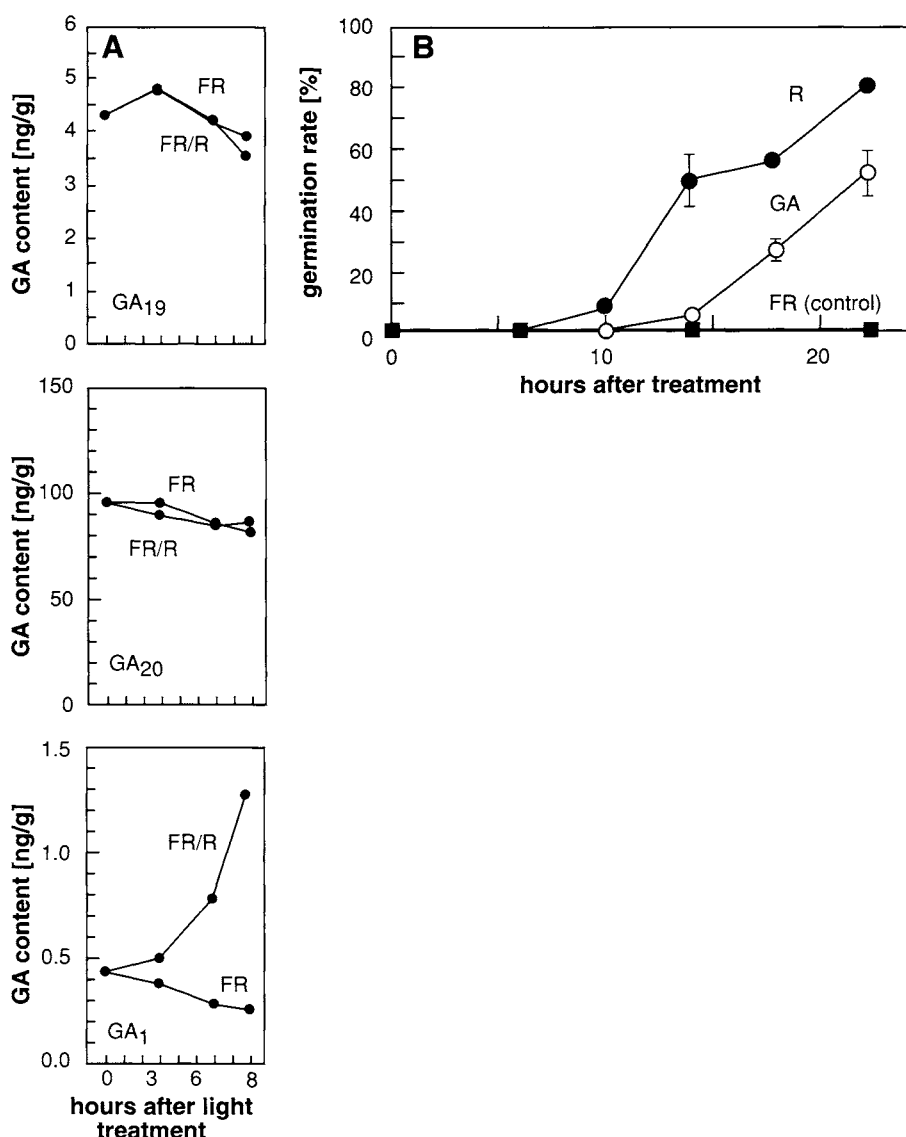


FIGURE 26-39 Effect of red light treatment on GA levels and effect of GA treatment on seed germination in lettuce (*Lactuca sativa* cv Grand Rapids) seeds. (A) Effect of red (FR/R) and FR (control) light treatments on endogenous levels of GA₁₉, GA₂₀, and GA₁. Note the difference in scale for the three GAs, and the sharp rise in GA₁ levels with FR/R treatment. From Toyomasu *et al.* (1993) with permission. (B) Seed germination rate following R, FR, and GA treatments. Note that the GA treatment almost completely bypasses the red light requirement for germination. For both A and B, seeds were imbibed for 3 h, then irradiated with FR light ($4.6 \text{ W} \cdot 10^{-2} \text{ m}^{-2}$, 10 min) or FR light followed by R light ($5 \text{ W} \cdot \text{m}^{-2}$, 10 min), or given GA treatment by exchanging solutions. A high concentration of GA₁ (2 mM) was used because seeds were not punctured. (Puncturing facilitates GA entry and seeds require only $\sim 1 \mu\text{M}$ GA₁). Except for light treatments, seeds were kept in darkness. From Toyomasu *et al.* (1994a).

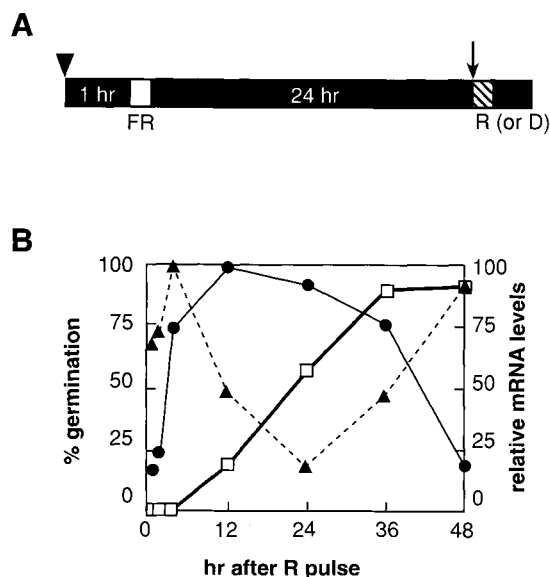


FIGURE 26-40 Red light-induced expression of *GA4* and *GA4H* genes and germination of seeds in *Arabidopsis*. (A) The protocol for light treatments. The seeds were imbibed in the dark for 1 h and then irradiated with a FR light pulse. Subsequently, the seeds were kept in darkness for 24 h and then either irradiated with a red light (R) pulse or incubated without the R light pulse in the dark (D). The triangle indicates the starting time of imbibition. The vertical arrow indicates the beginning of the R light pulse, which was set as 0 h in the graph in B. (B) Seed germination frequency (□) and the levels of *GA4* (▲) and *GA4H* (●) mRNAs after the R light pulse. For each data point of *GA4* or *GA4H*, 12.5 μ g of total RNA was hybridized with gene-specific probes, and the radiolabel in each blot was quantified and plotted. The highest mRNA levels for each gene were set as 100. From Yamaguchi *et al.* (1998).

1.1.2. Elongation Growth and De-etiolation

The interaction between phytochrome and gibberellin-induced elongation growth of epicotyls/hypocotyls of dicot seedlings and of mesocotyls in grass seedlings has been investigated intensively. Data show that light downregulates gibberellin-induced elongation, but whether it does so by a change in the content of active GA, by reducing the sensitivity of stem tissues to GA, or both is not clear.

Evidence that the activated phytochrome(s) inhibits synthesis of bioactive GAs in stems/petioles is mostly indirect. For example, transgenic plants overexpressing *PHYA* or *PHYB* are dwarfs when grown in light (see Fig. 26-23), and tobacco plants overexpressing oat *phyA* are reported to contain lower levels of GAs than untransformed plants. Pea seedlings, cv Alaska, grown under different fluences but the same spectral quality of white light show differences in height, as expected, which roughly correlate with the GA_1 content (Fig. 26-41).

A decline in GA_1 content on de-etiolation has been reported for lettuce seedlings. A study on pea (*Pisum*

sativum) seedlings cv Alaska grown in dark and then transferred to light showed a sharp drop in GA_1 content within 2 h of transfer (Fig. 26-42), although no significant changes were seen in other GAs (the precursor GAs, GA_{44} , GA_{19} , GA_{20} , or the inactivated metabolites, GA_8 or GA_{29}) for 24 h.

Whether the decline in GA_1 levels is via phytochrome action remains unclear. In the study on pea seedlings just referred to, the expression of two genes, a *GA 20-ox* and a *GA3 β -hy*, was studied using two mutants that were specifically deficient in *phyA* or *phyB* (*fun1-1* or *lv-5*, respectively, see Table 26-3). It could be shown for apical bud growth and expansion of leaves, which are part of the de-etiolation response in seedlings, that transcription of the *GA 20-ox* gene was upregulated by *phyB* as well as *phyA*. In contrast, expression of the *GA3 β -hy* transcripts was not responsive to phytochrome, although the transcripts accumulated to a higher level in stem tissues

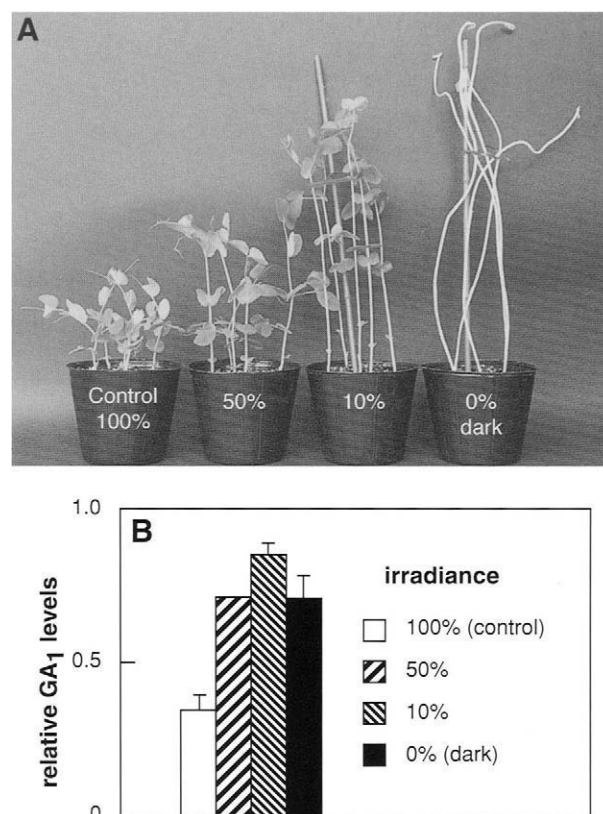


FIGURE 26-41 (A) Alaska pea (*Pisum sativum*) plants grown for 8 days at high irradiance $387 \pm 70 \mu\text{mol m}^{-2} \text{s}^{-1}$ (control 100%), medium irradiance (50%), low irradiance (10%), or in darkness (0%). (B) Endogenous GA_1 levels in the same plants. GA_1 content was 2 to 2.4-fold higher in medium and low irradiance plants than in the control. They also had high levels of GA_{20} (not shown). From Gawronska *et al.* (1995).

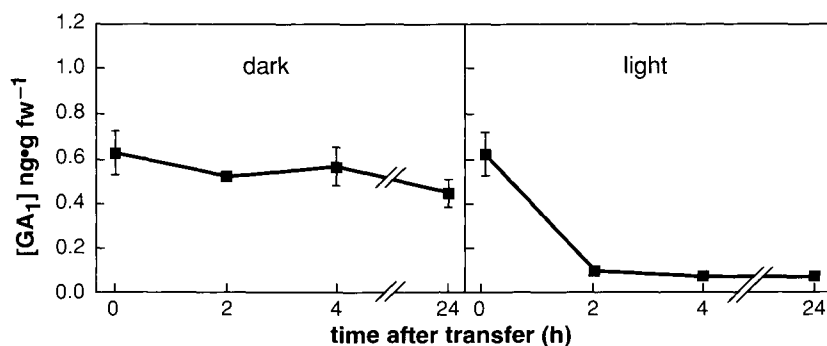


FIGURE 26-42 Endogenous GA₁ levels in pea epicotyls during de-etiolation. Seedlings were grown for 6 days in darkness and then either kept in darkness or transferred to light. Samples were taken and GA levels measured at indicated times. Zero hour represents the time of transfer to light. Data for GA₁ only are shown. Modified from Ait-Ali *et al.* (1999).

than in apical bud, and more so in dark than in light. Thus, it remains unclear which GA regulatory genes involved in elongation growth are affected by light. It is possible that other *GA20-ox* or *GA3b-hy* genes exist in pea which encode isoforms that are involved in the light-mediated downregulation of GA₁ content in stem tissue. Alternatively, light may activate a GA2β-hydroxylase, which lowers the content of bioactive GA in light.

Phytochrome action also seems to affect the sensitivity of stem tissues to applied GAs. The *phyA* or *phyB* mutants, especially the latter, show an elongated phenotype, similar to plants treated with exogenous GAs, or to that of the constitutive GA response mutants (e.g., *la cry*^s pea, *slender barley*, see Chapter 24). Many of these *phyB* mutants (e.g., *phyB* in *Arabidopsis*, *lh* in cucumber, *lv* in pea) have about the same endogenous content of GAs as the wild type. These mutants still respond to exogenous GA, which means that in a *phyB* mutant background, stem tissues show greater sensitivity to exogenous GAs. This is shown elegantly by a double mutant between a null mutant of *phyB* and the GA biosynthesis mutant, *ga1-3* in *Arabidopsis*, which is blocked in *ent-kaurene* biosynthesis and produces very little endogenous GA. The double mutant *phyB ga1-3* given exogenous GA elongates approximately three times more than the single mutant *ga1-3* (Fig. 26-43). It should be noted, however, that some mutants tentatively identified as *phyB* types, e.g., *ma*₃^R in *Sorghum* and *ein* in *Brassica*, are reported to have elevated levels of GAs.

In dark-grown grass seedlings, mesocotyl growth is arrested but coleoptile growth is favored. In rice seedlings supplied with exogenous GA, mesocotyls elongate, an elongation that is suppressed by red light of low fluence. The phytochrome-mediated inhibition of GA-induced mesocotyl growth is thought to result

from a decline in sensitivity to exogenous gibberellins, but the details are unknown.

We do not know what factors are involved in enhanced (or reduced) “sensitivity.” Stem elongation growth, as we have seen in Chapter 15, is a complex response brought about by loosening of the cell wall (by enzymes such as expansins, XETs, and endo-1, 4-β-glucanases) and enhanced turgor pressure. It also involves cytoskeletal- and plasma membrane-related changes, as well as developmentally regulated changes

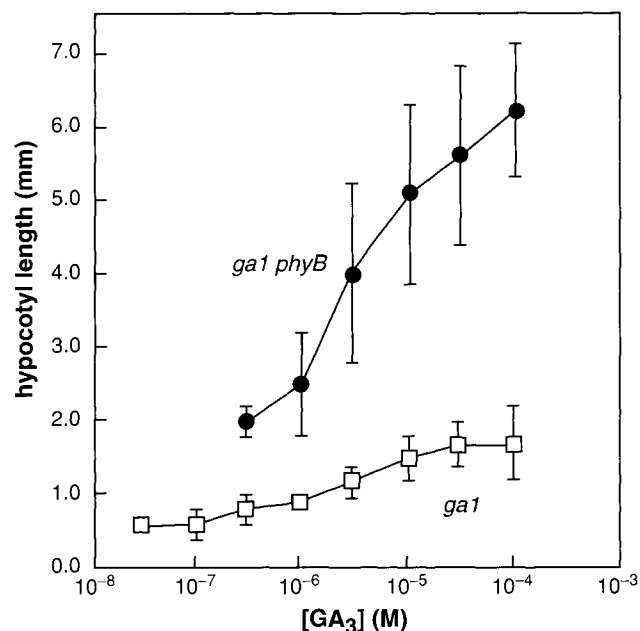


FIGURE 26-43 Hypocotyl elongation of *ga1-3* and *ga1-3 phyB-5* *Arabidopsis* seedlings in response to exogenous gibberellin A₃. Both mutants elongate in response to exogenous GA, but the double mutant responds about three times more than the single mutant at similar GA concentrations. From Reed *et al.* (1996).

in cell wall composition. Whether phytochrome action affects any of these parameters is unknown.

In summary, phytochromes and GAs interact to regulate several physiological responses. Both phyA and phyB (and possibly other type II phytochromes) are involved. Some responses are synergistic, e.g., seed germination where phytochrome action brings about synthesis of active GAs. Similar synergism may also prevail in the part of the de-etiolation response dealing with apical bud growth and leaf expansion. In other responses, e.g., stem elongation growth, the action may be antagonistic. The light signal seems to act at or before the transcription of genes encoding enzymes in GA biosynthesis (Fig. 26-44). In stem elongation, it may also act on other aspects of growth that alter the sensitivity of stem tissues to GA.

In a comprehensive study using *Arabidopsis* plants doubly homozygous for GA-related and phytochrome-related mutations, it was found that for a full expression of *phyB* mutant phenotype, an intact GA synthesis and signaling system is required. For example, the elongated phenotype of the *phyB* mutant is suppressed in a GA-deficient or -insensitive background. Thus, GA signaling also impinges on phytochrome signaling. In Chapter 24 it was noted that the elongation growth of stems is regulated negatively by GAI/RGA-type proteins and that the exogenous supply of GA derepresses this regulation. The connection, if any, between phytochromes and these negative regulators is unknown.

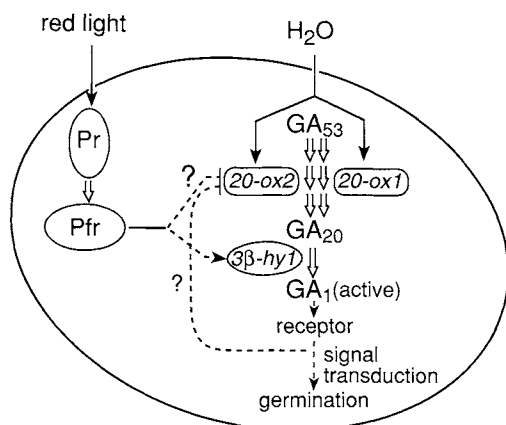


FIGURE 26-44 A model showing the interaction between phytochrome and GA signaling in the control of lettuce seed germination. In the imbibed seed, phytochrome in the Pfr form is shown activating a GA 3 β -hydroxylase (GA3 β -hy) gene, while inhibiting a GA 20-oxidase (GA20-ox2) gene. GA synthesis, however, proceeds *via* another GA 20-oxidase (GA 20-ox1) gene, which is not subject to phytochrome regulation. From Toyomasu *et al.* (1998).

TABLE 26-8 Free IAA in Epidermal Peels and the Apical Segment (1 cm) of Mesocotyl of Dark-Grown (D) and Red Light-Irradiated (R) Maize Seedlings^a

Tissue	Dry weight (mg)	IAA (ng · g dry weight ⁻¹)
D apical 1 cm	39.4 ± 0.006	508.0 ± 125.3
R apical 1 cm	41.6 ± 0.002	359.3 ± 90.6
D epidermis	26.6 ± 0.003	303.9 ± 76.1
R epidermis	36.0 ± 0.002	163.7 ± 41.5

^aBarker-Bridgers *et al.* (1998).

1.2. Phytochrome and Auxin Interaction

Several studies have indicated an interaction between light and auxin in the regulation of elongation growth. Thus, basipetal transport of IAA is necessary for hypocotyl elongation in light-grown but not dark-grown seedlings of *Arabidopsis*. In dark-grown pea seedlings, elongation was suppressed by irradiation with R light, and it was suggested that the suppression resulted from the depletion of IAA concentration not in the whole stem, but selectively within the epidermis. As shown in Chapter 15, epidermal cell walls control the rate of extension growth of axial organs. In a study on maize seedlings, highly sensitive methods for measuring picomole quantities of IAA were used. Data confirmed the decline in the growth rate of mesocotyl after R irradiation and related it to a decline in the IAA content of mesocotyl, particularly in epidermal cells (Table 26-8). Thus, IAA levels are indeed affected by red light, and IAA has a particular and specific effect on the epidermal cell walls.

A gene encoding a homeodomain-leucine zipper (HD-Zip) protein in *Arabidopsis* (*ATHB-2*) is associated with shade avoidance syndrome. The gene is expressed in seedlings and established plants at low levels under high R:FR ratios, but is induced rapidly and strongly if the R:FR ratio is lowered. In *phyB* mutants, this relationship is lost. Transgenic plants overexpressing the gene and exposed to a low R:FR ratio show enhanced hypocotyl elongation, which is curtailed by use of the auxin transport inhibitor, naphthylphthalamic acid (NPA). Thus, it is thought that shade avoidance responses, such as enhanced apical elongation, reduced cambial growth, and lateral rooting, are brought about by changes in auxin gradients or transport. How the *ATHB-2* protein modulates auxin transport or sensing mechanisms is unknown.

More support for an interaction between phytochrome and auxin signaling is provided by the cloning of the *SHY2* gene. *Arabidopsis hy2* is a phytochrome chromophore mutant that displays deficiency in all phytochrome

mes and an elongated phenotype in light. Dominant suppressor mutants known as *shy1* and *shy2* (for suppressor of *hy*), which suppress the elongated hypocotyl phenotype of *hy2* mutants, were obtained. The *shy2* mutations predominantly affect auxin-dependent root phenomena, such as lateral root formation and gravitropism. The *SHY2* gene has been cloned and is identical to *IAA3*, one of the members of the Aux/IAA gene family (see Chapter 22). The gene is now known as *SHY2/IAA3*. Both *ATBH-2* and *SHY2* provide examples of the interactions that prevail between light and auxin signaling in the control of complex processes such as shade avoidance, hypocotyl elongation, and root growth.

1.3. Photomorphogenesis of Dark-Grown Seedlings

Two hormones, brassinosteroids and cytokinins, play opposite roles in the de-etiolation response of seedlings. Brassinosteroids are essential for the maintenance of etiolated habit in the dark because *Arabidopsis* mutants deficient in brassinosteroid biosynthesis (e.g., *det2*, *cpd*) or insensitive to BRs (e.g., *bri1/cbb2*) show the de-etiolated phenotype of the *det/cop/fus* mutants with the important exception that they are not seedling lethals. A supply of exogenous brassinolide (BL) or castasterone to BR-deficient (but not -insensitive) mutants grown in dark restores them to the etiolated phenotype. These observations suggest that, in the dark, brassinosteroids operate in a separate pathway to maintain the etiolated habit in the dark. In the light, brassinosteroids enhance stem growth. Since the *det2* mutant grown in light is disrupted in its photoperiod and timing responses, BRs may interact with photoreceptors, but such interaction has not been studied. Many genes encoding enzymes involved in brassinosteroid synthesis have been cloned (see Chapter 9), and light does not appear to regulate the activity of these genes. In fact, these genes have similar, ubiquitous expression throughout the plant. Likewise, the expression of *BRI1*, the gene encoding a transmembrane kinase thought to be a receptor for BRs (see Chapter 24), is unaffected by light.

The mutation *bas1-D* (for phyB activation-tagged suppressor 1-dominant) has been isolated using the mutagenesis protocol for activation tagging (for this protocol, see Appendix 1). The wild-type gene *BAS1-D* encodes a cytochrome P450, called BAS1, that is thought to catalyze the inactivation of BL by 26-hydroxylation (for inactivation of BRs, see Chapter 9). The mutant plants are dwarfs, lack BL, and accumulate 26-OHBL. Epistasis analysis using double mutants of *bas1-D* with null alleles of *phyA*, *phyB*, or *cry1* indicates

that BAS1 acts downstream of both *phyA* and *cry1* while it suppresses the *phyB* phenotype. Thus, BAS1 seems to act at one of the control points between multiple photoreceptor systems; it also suggests that brassinosteroid signaling may be modulated by BR inactivation.

1.4. Light and Cytokinin Interaction

The action of cytokinins is opposite to that of brassinosteroids. Exogenous cytokinins supplied in supraoptimal concentrations to dark-grown seedlings cause de-etiolation, including short hypocotyls, open expanded cotyledons, development of chloroplasts, and expression of photosynthetic genes (*CAB*, *RBCS*), as well as genes involved in anthocyanin biosynthesis, such as *CHS* and *DFR*. At least part of the de-etiolation program, short swollen hypocotyls, is brought about by the cytokinin-induced synthesis of ethylene (see Chapter 24). The cytokinin-overproducing mutant *amp1* (for altered meristem program) of *Arabidopsis* has a striking similarity to *det/cop/fus* mutants, but shows some unique features as well, such as multiple cotyledons.

Light and cytokinins bring about de-etiolation and induction of genes probably by independent pathways, which converge at some point prior to gene induction. This is supported by the observation that CK-induced ethylene production at high CK concentrations is severely curtailed in light relative to that in dark.

The interaction between light and cytokinins extends further. Cytokinins, such as benzyladenine, inhibit transcription of the *PHYA* gene, as does light. Also, the *det1* mutant and the brassinosteroid biosynthesis mutant, *det2*, show enhanced sensitivity to cytokinins both in prevention of senescence in detached leaves and in tissue culture. The cytokinin levels in *det1* and *det2* mutants, however, are similar to those in the wild types.

1.5. Light and Abscissic Acid

A light-induced decrease in ABA content has been reported in several plants and plant organs (e.g., *Lemna gibba* fronds, tobacco plants, lettuce, and pine seeds) and attributed to phytochrome action. For example, in *Lemna* fronds, this decrease was shown to be R/FR reversible. It is not clear, however, whether the decrease results from an inhibition of ABA synthesis or ABA inactivation by oxidation to phaseic acid or conjugation. ABA and phytochrome are also known to regulate certain genes in an antagonistic manner (e.g., the *Em* gene in wheat embryos and *RBCS* and *CAB* genes in *Lemna*). In *Lemna*, it could be shown, however,

that R light and ABA acted independently in regulation of a *CAB* gene. Thus, while phytochrome downregulates ABA content in plant tissues, there seems to be no direct interaction between phytochrome and ABA signaling. Significantly, negative regulation of the *CAB* gene in *Lemna* by ABA was not reversed by gibberellin treatment.

1.6. Induction of Flowering

Flowering is a highly complex response that involves integration by the plant of developmental cues and biological clock with environmental signals, such as daylength and temperature. Photoreceptors, especially phytochromes, and hormones, particularly gibberellins, are involved. Thus, *phyB* mutants typically flower earlier than wild-type plants.

Treatments that enhance endogenous GA content also lead to flowering or to early flowering in long-day plants (LDs). Thus, in *Silene armeria*, an LD plant, transfer from short days (SD) to LD results in a severalfold increase in the levels of GA₁, particularly in the subapical region. In rosette plants, such as spinach (*Spinacea oleracea*) and *Arabidopsis*, induction of flowering by LDs is correlated with an increase in GA content. In spinach, this increase is related to an increase in GA-20 oxidase mRNA levels. Conversely, GA-deficient dwarf mutants of *Arabidopsis* flower late in LDs and fail to flower in SDs.

These correlations between LDs, increased GA content, and flowering suggest, but do not prove, that the flowering response under LDs is related to altered ratios of Pfr/P. It is possible, however, that in the flowering response, the activated phytochromes may delay flowering in the natural environment by downregulating GA levels at the GA-20 oxidase level. In stem growth, as mentioned earlier, activated phytochrome may downregulate endogenous GA content, whereas in seed germination, it may increase GA levels.

2. SECTION SUMMARY

Several hormones, particularly gibberellins, indole acetic acid, brassinosteroids, and cytokinins, interact with phytochromes to regulate several processes. Phytochrome signaling interacts with gibberellin signaling in seed germination, de-etiolation, stem elongation, and flowering response. It seems that while the activated phytochrome enhances the synthesis of biologically active 3 β -hydroxylated GAs in seed germination and apical bud growth and leaf expansion

during de-etiolation, it downregulates GA synthesis or sensitivity in stem elongation, as well as in the flowering response under LDs. The likely sites of such regulation in seed germination and de-etiolation responses appear to be genes encoding GA 20-oxidases, GA 3 β -hydroxylases, and possibly GA-2 β -hydroxylases. Phytochrome in the Pfr form is also thought to inhibit IAA-induced elongation growth, possibly by reducing IAA content in epidermal cells. Moreover, two genes, *ATBH-2* and *SHY2*, in *Arabidopsis* provide common links between phytochrome signaling and IAA action or transport. Brassinosteroids are involved in the maintenance of an etiolated phenotype in dark-grown seedlings, but in light-grown plants, they promote elongation growth and, like GAs, counter the light inhibition of elongation growth. BRs may act at a common point where signaling pathways from several photoreceptors converge. The cytokinins, at supraoptimal concentrations, bring about the de-etiolation response in dark-grown seedlings. They probably operate in a parallel but independent pathway to that of phytochromes and cryptochromes in mediating de-etiolation in light.

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3. Discovery of Phytochrome

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SECTION I. PHYTOCHROMES AND CRYPTOCHROMES

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SECTION IV. PLANT HORMONES AND LIGHT SIGNALING

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Tropic and Nontropic Responses to Environmental Signals

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1. PLANT RESPONSES TO ENVIRONMENTAL STIMULI OFTEN INVOLVE MOVEMENT

Plant responses to external stimuli, such as light, gravity, touch, and water, and to endogenous developmental signals set by the biological clock often involve movements. Some are growth movements, others are nongrowth movements, and some have components of both. Movements may also be in response to a directional stimulus (i.e., a tropic movement) or a stimulus that is diffuse (nastic movement).

Common examples of growth movements resulting from a directional stimulus are phototropism and gravitropism. Other growth movements may occur in response to mechanical stimuli, such as touch (thigmotropism), or sensing of and growth toward water (hydrotropism) or chemicals (chemitropism), including oxygen (oxytropism). Some growth movements occur in response to a diffuse stimulus and hence are considered nastic movements. For example, shoot tips often describe an oscillatory movement as they grow upward, a movement known as circumnutation. Leaves droop or show an epinastic curvature in their petioles; sometimes they curve upward and show hyponasty.

Nongrowth movements occur in mature parts of the plant. Most of these movements occur in response to directional or diffuse light. Others occur in response to mechanical stimuli, including touch. The best and most common example of nongrowth movement is that provided by stomata. Stomatal pores open and close by reversible turgor changes in guard cells and are regulated by a number of environmental factors, including blue light, as well as by hormones such as ABA. Other examples include movement of chloroplasts in leaf cells of *Funaria* (a moss) and *Lemna* (duckweed), and leaf reorientations (e.g., *Erythrina* spp. and *Phaseolus vulgaris*), and "rolling" of lamina (e.g., grasses) to avoid excessive solar radiation and transpirational loss. Leaves and flowers of many plants show what are known as "sleep" movements. They open or unfold with sunrise (photonasty) and close or fold with sunset (nyctinasty). These diurnal movements are common in compound leaves of members of Fabaceae and Oxalidaceae. Flowers of tulip (*Tulipa* spp.) close their petals at night and open them in the morning.

Nastic leaf closure may also result from mechanical stimulation or touch (e.g., the sensitive plant, *Mimosa pudica*, and insectivorous plants, such as Venus fly trap).

A special kind of directional movement, which may involve growth, occurs in solar tracking (heliotropism). The phenomenon is common in many herbaceous plants belonging to diverse families of angiosperms and is shown by mature leaves or flowers.

This chapter deals mainly with phototropism and gravitropism, and emphasis, as in Chapter 26, is on seed plants, especially angiosperms. Interesting aspects of some movements other than those associated with photo- or gravitropism are presented in the Box 27-2. Blue and red light-mediated responses also occur in spore-bearing plants (cryptogams); indeed, available data indicate that the photoreceptors in these plants are more complex and variable than those in seed plants. These responses are covered briefly in Box 27-1.

2. PHYSICOCHEMICAL BASES FOR MOVEMENTS

All movements, growth or nongrowth, whether they are due to an external or an internal signal, involve perception of stimulus by a receptor, transduction of that signal *via* secondary messengers or an electrical gradient to the response site, and conversion of the message to the overt response. As will be evident, many of these responses are mediated by directional or diffuse light; hence, the receptors in these cases are likely to be phytochromes and/or B/UV-A photoreceptors; little is known about UV-B photoreceptors. Gravitropism involves perception of gravity, which, like touch, may be considered as perception of pressure.

Tropic responses, such as phototropism and gravitropism, are obtained only in growing parts of a stem or root; hence, genes involved in cell growth are activated, and the necessary turgor and wall-loosening factors are already in place. The stimulus of light or gravity changes the direction of growth by changing the orientation of microfibrils, which may be preceded by a change in orientation of the cytoskeleton and/or alterations in the local properties of plasma membrane. Indoleacetic acid (IAA), the endogenous auxin, has been postulated for a long time as mediating phototropic and gravitropic curvatures. This role of auxin in tropic growth has been confirmed in recent years by the discovery of mutants (in *Arabidopsis*) that are impaired in auxin uptake/transport and/or signaling.

Although they involve different perception mechanisms and signaling intermediates, phototropism and gravitropism culminate in the same response— asymmetric growth of root or shoot, which is mediated, at least in part, by auxin. The following two sections, therefore, consider the features of phototropism and gravitropism that are unique to each response, including the mechanism of perception and signaling. A third section deals with the phenomenon of asymmetric growth, the role of auxin in that growth, and other hormones, especially ethylene, which participate with auxin in asymmetric growth.

SECTION I. THE PHOTOTROPIC RESPONSE

In the natural environment, shoots bend toward a light source because they perceive a difference in light quality and/or quantity on two sides of the stem and respond by differential growth on the two sides. Also,

the light signal is perceived continuously for some time. A dissection of the phototropic response in the laboratory, however, usually employs etiolated materials and exposure to short durations of light. These studies have revealed that the phototropic response, while being one of the most sensitive responses to light known — it can be triggered by very low fluences of light—is also quite complex.

1. CHARACTERISTICS OF PHOTOTROPIC RESPONSE

1.1. Fluence–Response Curve for Phototropism

If etiolated coleoptiles or epicotyls/hypocotyls are irradiated with pulses of light of increasing fluence, the response begins after a lag period of 8–10 min, rises to a peak, and then declines (Fig. 27-1A). It may even become negative before a second rise. The response plotted as a fluence–response curve shows the first rise and decline, also known as the ascending and descending arms of the first positive phototropism followed, after a period of no, or even negative, re-

sponse, by a second rise, which is referred to as the second positive phototropism. The response curve for the first positive phototropism in all plants investigated is a symmetric, bell-shaped curve and obeys the law of reciprocity over a relatively wide fluence range, about three to four orders of magnitude (Fig. 27-1B, for law of reciprocity, see Box 26-1 Chapter 26). The fluence requirement to obtain maximal curvature is small, with the response peaking at a fluence of $< 1.0 \mu\text{mol m}^{-2}$ (see Fig. 27-1B). In contrast, the second positive phototropism, does not obey the law of reciprocity; it is a high irradiance response that requires exposure of long durations and is time dependent, not fluence dependent. It is also affected by temperature.

In the natural environment, in most cases, the phototropic response occurs according to the second curvature because the fluence at the bottom end of the descending arm of the first positive curvature ($\sim 10 \mu\text{mol m}^{-2}$) can still be saturated by a few seconds exposure to sunlight. In nature, probably, the two curvatures blend into one. The first and second phototropic curvatures explain some important characteristics of the perception of light and the nature of the photoreceptor(s) involved. In order to

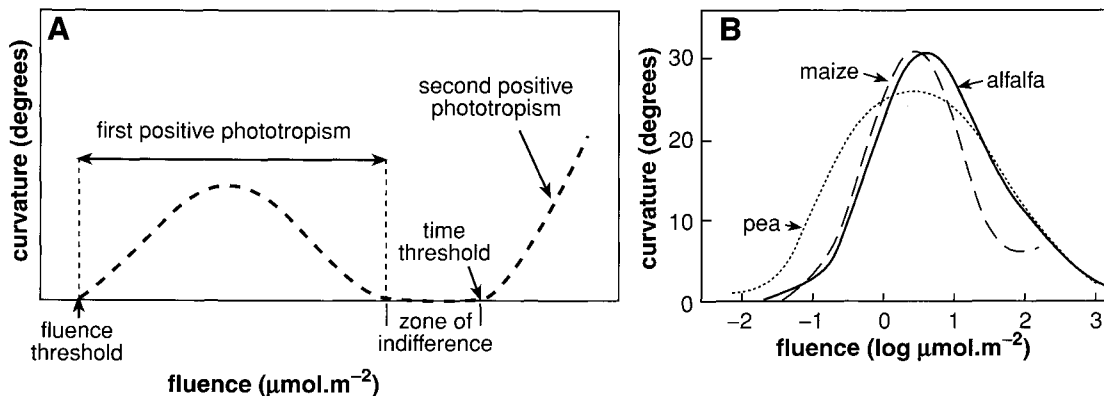


FIGURE 27-1 Fluence–response curves for phototropism. (A) Idealized drawing of the phototropic response with increasing fluence. From Janoudi and Poff (1993). (B) Fluence–response curves for first positive phototropism for three plants; alfalfa (*Medicago sativa*), pea (*Pisum sativum*), and maize (*Zea mays*). For phototropic stimulation, dark-grown seedlings were irradiated with pulses of unilateral blue light ($\sim 450 \text{ nm}$) of increasing fluence. Curvature was measured from 90 to 120 min after the blue light stimulus. Seedlings were grown in continuous red light at a low fluence rate to maintain a constant Pfr/P ratio throughout the experiment. Note: Etiolated coleoptiles and mesocotyl of grass seedlings are extremely sensitive to very low fluences of light and phytochrome does absorb part of the spectrum in blue-UV. Hence, it is possible that phytochrome may affect measurement of the phototropic response due to blue light. To eliminate such interference, blue light-mediated responses are often measured in etiolated materials that are grown and kept in continuous red light at a low fluence rate ($0.15 \mu\text{mol m}^{-2} \text{ s}^{-1}$). Under these conditions, the Pfr/P ratio is maintained at a steady level, which is not likely to be perturbed by absorbance in blue during the phototropic measurement. However, under Rc, the fluence response increases by approximately one order of magnitude. From Baskin and Iino (1987).

understand that, we must first look at the action spectrum of phototropism.

1.2. Action Spectrum for Phototropism

Action spectra can be plotted for the entire fluence range of the first positive phototropism and thus include both ascending and descending arms. As shown for alfalfa, there are no qualitative differences between the two plots (Fig. 27-2A). Hence, most authors plot the action spectrum for the ascending arm only. An action spectrum for the phototropic curvature for an etiolated oat coleoptile is shown in Fig. 27-2B and is similar to the action spectra for alfalfa. Spectra show a maximal response in the blue region (~ 450 nm) and strong shoulders at ~ 475 and ~ 425 nm. Such spectra, referred to as the "three-finger" pattern, are typical for photoresponses with maximal activity in blue region of the spectrum. This includes blue light-

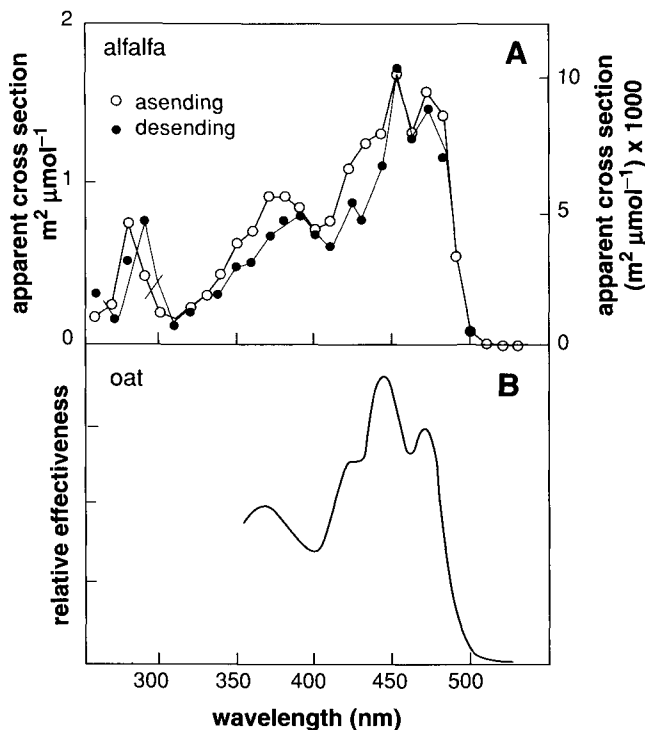


FIGURE 27-2 Action spectra for the first positive phototropism. (A) Action spectra for an etiolated alfalfa (*Medicago*) hypocotyl were plotted for the entire fluence range of the first positive phototropism and thus include both ascending and descending arms. The ordinate gives the reciprocal of the photon fluence required to give the standard response (a curvature of 13°). The scale on the left is for the ascending arm and the one on the right is for the descending arm. (B) Action spectrum from an etiolated oat (*Avena*) coleoptile. In both figures, spectra show maximum response in blue (~ 450 nm) and strong shoulders at ~ 475 and ~ 425 nm. From Baskin and Iino (1987).

induced responses mediated by cryptochromes and stomatal opening mediated by a yet unidentified receptor. Much lower shoulders occur in UV-A (~ 370 nm) and UV-B (~ 280 nm) regions. No response occurs above ~ 500 nm. Hence, red or far-red irradiations and phytochromes that absorb those radiations are excluded as primary receptors for phototropism. However, as shown later, phytochromes amplify the signal from a primary blue light photoreceptor.

1.3. The Nature of the Blue Light Photoreceptor

In plants, the major pigments absorbing in the blue and UV regions of the visible spectrum are carotenoids, e.g., β -carotene, and flavonoids, e.g., riboflavin (Fig. 27-3). Both carotenoids and flavins have been postulated for a long time to be the chromophore involved in phototropism, but for various reasons those ideas were not entertained seriously. For example, Arthur Galston at Yale University hypothesized as early as 1949 that the chromophore involved in phototropism was a flavin, but Galston's hypothesis to explain the phototropic curvature coupled the absorption of blue light by a flavoprotein to selective destruction of auxin on the irradiated side. Since a differential destruction of auxin on the lighted side did not occur, in a classic case of the baby being thrown out with the bath water, the whole idea was rejected. The issue remained controversial until 1998 when an *Arabidopsis* mutant defective in the phototropic response led to the cloning of the gene for a putative receptor and supporting evidence that it carries a flavin prosthetic group as the chromophore.

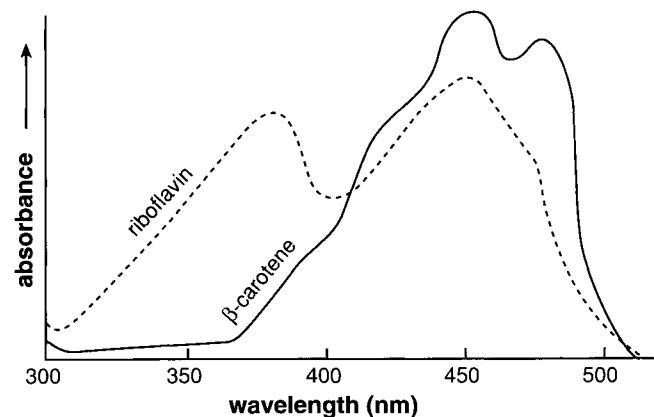


FIGURE 27-3 Absorption spectra for riboflavin and β -carotene. From Wareing and Phillips (1981).

1.4. Phototropic Response Involves More Than One B/UV-A Photoreceptor

Since there are no qualitative differences between ascending and descending arms of the first phototropic response, it may be hypothesized that a single photochemical step mediates the response at the entire fluence range. However, it has been shown that the shape of the fluence–response curve for first phototropism is affected by the fluence rate, as well as the wavelength of the actinic beam. For example, a symmetrical curve is obtained and reciprocity holds over the fluence range only if fluence rates are kept low (0.1 to $0.2 \mu\text{mol m}^{-2} \text{s}^{-1}$). If blue light pulses at higher fluence rates (0.4 – $0.7 \mu\text{mol m}^{-2} \text{s}^{-1}$) are given, two maxima may be seen, or if a light pulse of $\sim 500 \text{ nm}$ is used after the pulse at 450 nm , the second peak may be canceled. These observations suggest the presence of at least two blue light photoreceptors: one acting at low fluences and the other at higher fluences. The idea of two blue light photoreceptors is also supported by fluence–response curves obtained with mutants defective in phototropic responses.

1.5. Photosensory Responses Are Subject to Adaptation

“Adaptation” in photosensory responses refers to the process whereby organisms undergo changes in their sensitivity and/or responsiveness to a light stimulus. In human vision, it is common to experience a “blinding flash,” which results in a transient loss of vision, or desensitization, after exposure to a pulse of high-intensity light. After a period of recovery, the vision is restored. Likewise, after being in the dark for a while, our eyes adapt to darkness and see objects that they were unable to see before dark adaptation. The phenomenon of adaptation is known for photosensory responses of plants and many other aneural organisms, although it has not been investigated to the same extent as in vertebrates or invertebrates. Nonetheless, the main features of adaptation, i.e., desensitization, recovery, and enhancement, all are known for photosensory responses in plants.

Adaptation may be at the sensor level or at the effector level. Sensor level adaptations include changes in concentration or sensitivity of the photoreceptor(s) and thus lead to enhancement of the signal or its attenuation (desensitization). The three to four orders of magnitude enhancement in sensitivity to red light in seeds in the soil bank after prolonged dark storage most likely is a case of adaptation at the sensor level (see Chapter 26). The effector level adaptations may be at any step in signaling down to the level of overt

response. In this case, the signal is perceived but the degree of output (responsiveness) may be attenuated or amplified. Examples of effector level adaptation are also common and are given later.

In experiments with etiolated seedlings, pulses of blue light enhance as well as desensitize the subsequent phototropic curvature induced by a pulse of unilateral blue light. The same fluences of blue light that bring about the first phototropic curvature also cause enhancement and desensitization. While enhancement can occur with other wavelengths of light (see section 1.6), desensitization occurs only with blue light. Using a desensitization protocol, it can be shown that the greater the fluence rate of blue light, the greater the degree of desensitization (Fig. 27-4) and longer the period of recovery. Thus, the fluence–response curve for first phototropism may be considered as a composite of enhancement and desensitization. In the ascending arm and at low fluences, enhancement exceeds desensitization. With increasing fluence, and at the point of maximal curvature, enhancement and desensitization are of equal magnitude. In the descending arm with increasing fluences, desensitization increases progressively in magnitude, reaching a maximum at the bottom end of the descending arm. If a flash of light at a fluence representing the fluence at the bottom end of the first curvature is given, no response is elicited. Following a refractory period, during which the level of responsiveness is constant, the plants recover and their phototropic responsiveness is enhanced. Second positive phototropism requires irradiation with blue light at higher fluences, but only if irradiation times exceed a time threshold; at increasingly higher fluence rates, increasingly longer irradiation times are needed to induce the second positive phototropism.

1.6. Phytochromes Amplify the Phototropic Response

Irradiation of etiolated stems/coleoptiles with unilateral blue light alone gives a curvature of only a few degrees, but preirradiation (bilateral or from above) with either red light or blue light causes an enhancement of curvature by a subsequent unilateral blue light. For example, in one study on *Arabidopsis* seedlings, blue light (450 nm at $0.5 \mu\text{mol m}^{-2}$) alone gave a small curvature ($\sim 10^\circ$), but preirradiation with increasing fluences of red light (667 nm) progressively increased the amplitude of curvature, which saturated at $\sim 10 \mu\text{mol m}^{-2}$ (Fig. 27-5).

Preirradiation with red light can also reduce the time threshold, or the width of the zone of indifference, before the second positive phototropic response.

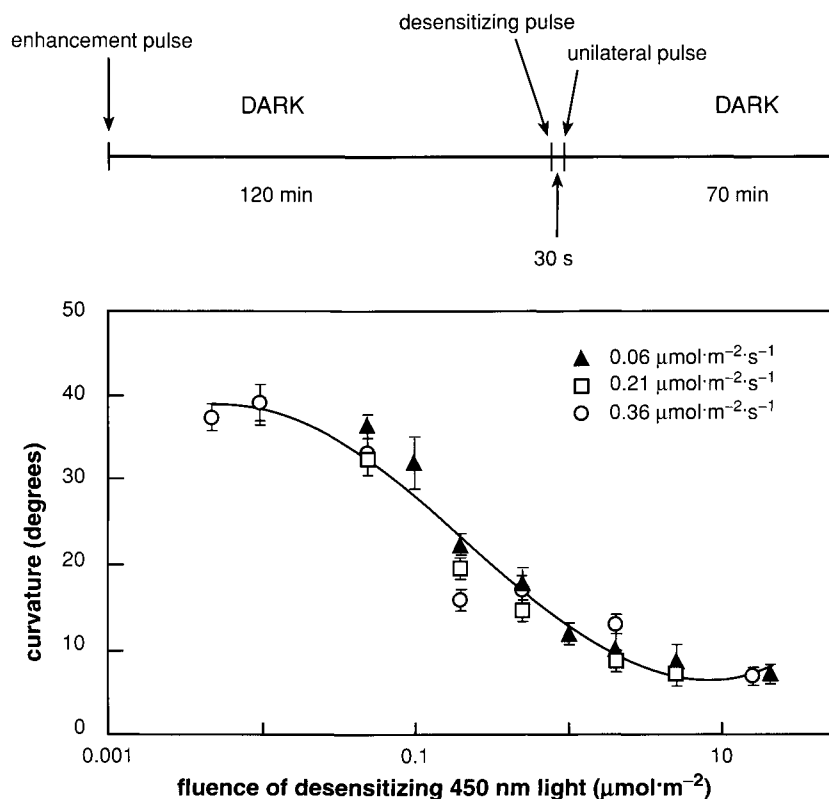


FIGURE 27-4 Desensitization of phototropic response by blue light. Etiolated *Arabidopsis* hypocotyls were exposed to pulses of blue light (450 nm) from two opposite sides (bilateral irradiation) as part of an enhancement procedure, followed by a period of 2 h of darkness and then given a pulse of desensitizing blue light (bilateral) followed within 30 s by a photoinductive pulse of unilateral blue light ($0.5 \mu\text{mol m}^{-2}$). The phototropic curvatures were measured 70 min after the unilateral blue light pulse. The graph shows the effects of increasing fluence of the desensitizing pulse on the subsequent curvature. The protocol for treatments is given above the graph. From Janoudi and Poff (1993).

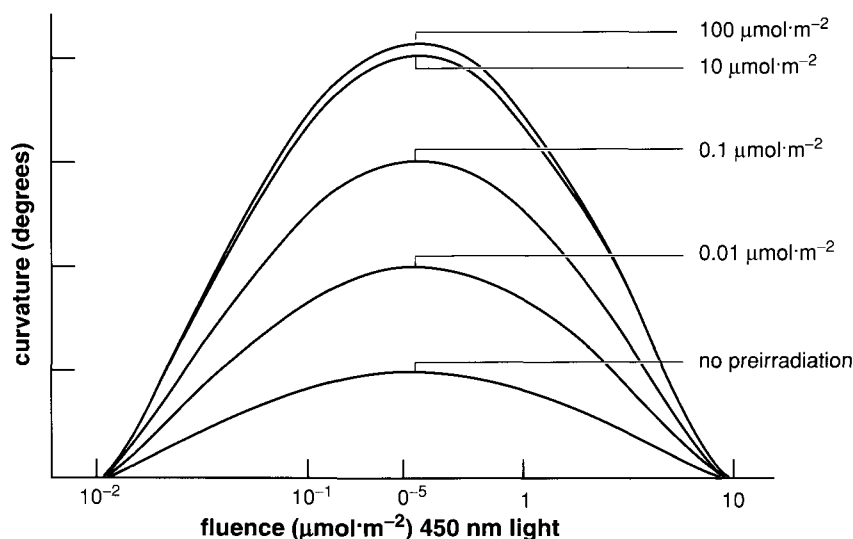


FIGURE 27-5 Enhancement of the magnitude of the first positive phototropic curvature by increasing fluences of red light (669 nm). Fluence-response relationships represent curvatures in response to irradiation with blue light (450 nm). Seedlings either received no red light preirradiation or a red light preirradiation at fluences indicated. Preirradiation with red light was nondirectional and was followed 2 h later with unidirectional blue light. From Janoudi *et al.* (1997).

These red light effects are brought about by phytochromes. As shown by the use of *phyA* and *phyB* mutants, both *phyA* and *phyB* are involved, but how they amplify the signal created by the blue light photoreceptor is unknown.

In summary, phototropism is a complex photosensory response in which adaptations at the sensor level as well as the effector level are seen. The response is triggered by irradiation with unilateral blue, green, or UV-A light, but not red or far-red light. The phototropic curvature first increases with an increasing fluence of blue light and then decreases and may even become negative before a second positive phototropism. Analysis of the phototropic response indicates that at least two blue light photoreceptors participate: one active at low fluences of blue light and the other active at higher fluences. In addition, phytochromes participate in the response, not as primary photoreceptors, but as amplifiers of the signal generated by the primary photoreceptors.

2. AXIAL ORGANS SHOW A GRADIENT OF LIGHT FLUENCE ON UNILATERAL IRRADIATION

Many authors have measured the internal light fluence rate in oat or maize coleoptiles subjected to unilateral illumination. In a careful study, dark-grown maize coleoptiles were irradiated unilaterally with

white light. Blue light (450 nm) fluences (scattered and collimated light) were measured radially across the coleoptile using a fiber-optic probe, which collected light only at its tip (5 μm diameter). The probe was driven at different heights in the coleoptile (bottom, middle, top) and at different angles: horizontally from the shaded towards the irradiated side or *vice versa*; or driven across the coleoptile at an angle to the horizontal. Bottom parts of the coleoptile included the first leaf; the tip lacked it. Results indicated that in the coleoptile, there was a steep gradient of blue light with the greatest amount of light on the irradiated side and falling off toward the shaded side (Fig. 27-6). The magnitude of the gradient was 4:1 between irradiated and shaded sides at the tip and the midregion; it was 5:1 at the bottom (because of the enclosed leaf).

3. PHOTOTROPIC MUTANTS IN *ARABIDOPSIS*

To isolate these mutants, mutagenized populations of etiolated seedlings are screened for phototropic responses at a range of fluences. The screen may utilize roots, which show negative phototropism, or above-ground parts, such as the hypocotyl/epicotyl, which show positive phototropism (Fig. 27-7A). Seedlings that do not respond or require a much higher fluence for the first phototropic curvature, but still give the same magnitude of response as the wild type,

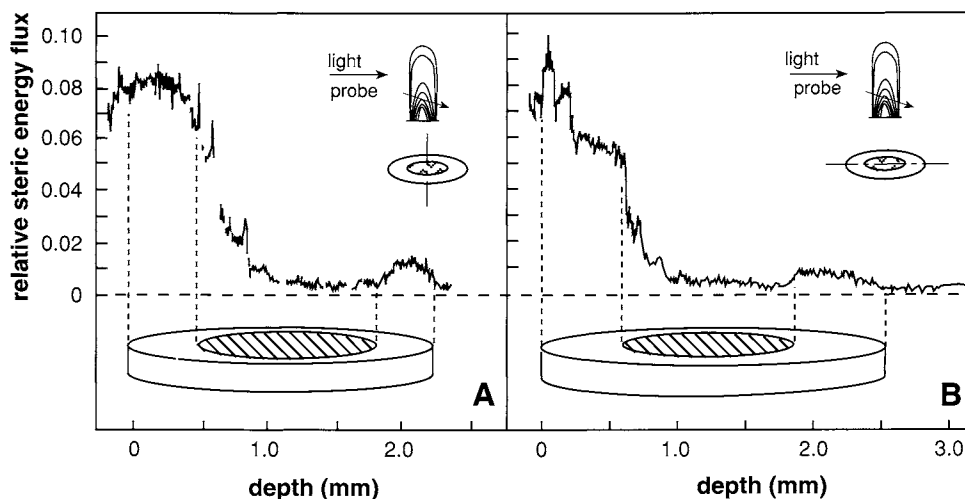


FIGURE 27-6 A representative blue light gradient in the basal region of a maize coleoptile. The coleoptile is ellipsoidal in cross section; it was positioned so that the thin (A) or the thick (B) profile of the coleoptile faced the light beam. In this experiment, the probe was driven near the base of the coleoptile at an angle (135°) from the lighted to the shaded side. In this sampling direction, mostly scattered light was collected by the probe tip. The direction of light and the angle and direction of the probe, and cross sections of the coleoptile together with the direction of light are shown in the insets. From Vogelmann and Haupt (1985).

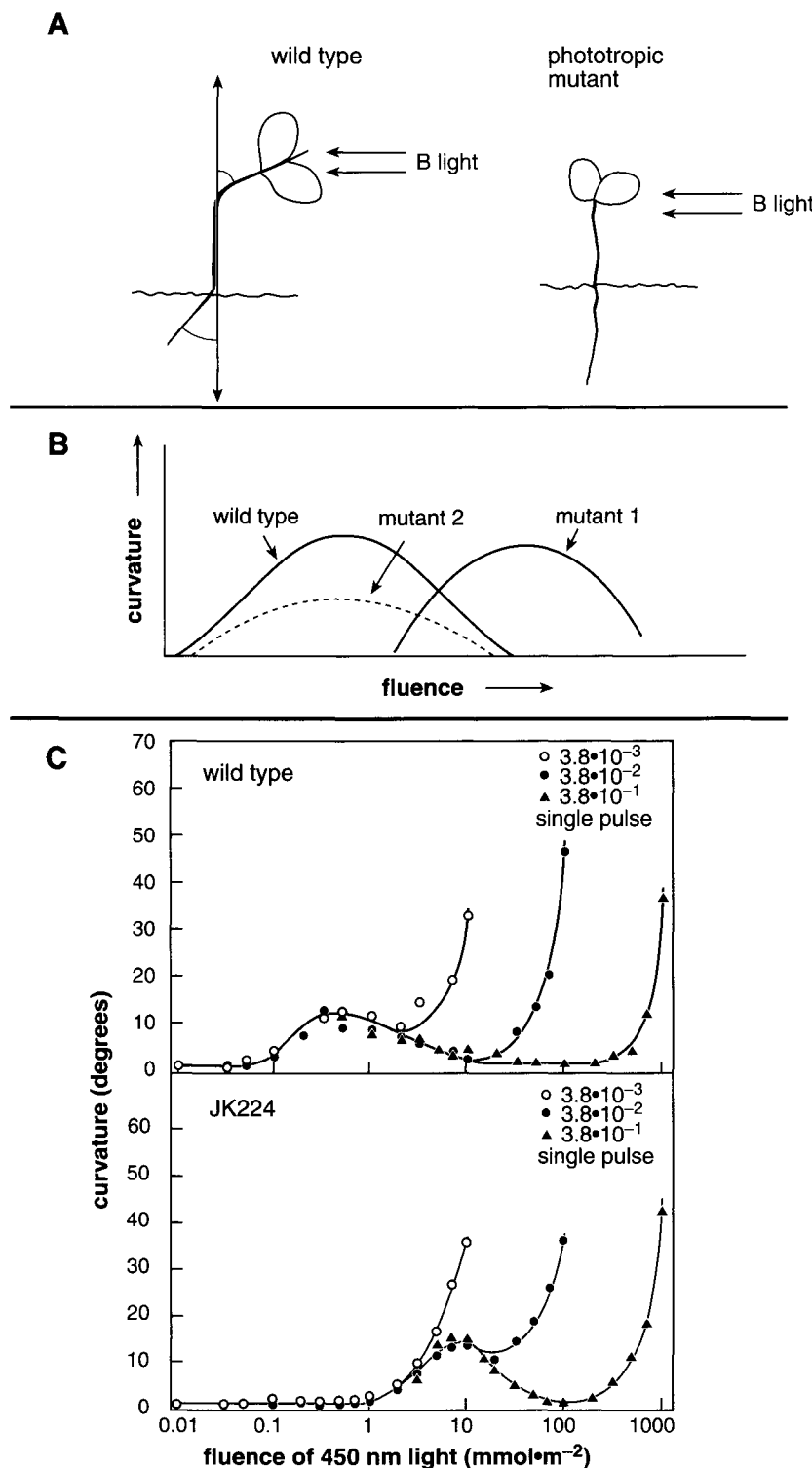


FIGURE 27-7 Screen for phototropic mutants in *Arabidopsis*. (A) A schematic drawing showing a wild-type seedling and a phototropic mutant both irradiated with unilateral blue light at an optimal fluence (e.g., $0.5 \mu\text{mol m}^{-2}$). In the wild type, shoots show positive phototropism while being negatively gravitropic. Roots show positive gravitropism and are also negatively phototropic; hence, in the wild type, they grow at an angle to the gravitational axis. In the mutant seedlings, roots, like shoots, show no phototropic response. (B) Fluence-response curves for first positive phototropism in hypocotyls/epicotyls of seedlings of the wild-type and phototropic mutants. Seedlings that show the same magnitude of response as the wild type but require

TABLE 27-1 Phototropic Mutants in *Arabidopsis*^a

Mutant	Site of action	Reference
<i>nph1</i> (for nonphototropic hypocotyl, includes JK224 and <i>rpt1</i>)	Photoreceptor	Khurana and Poff (1989); Okada and Shimura (1992); Sakai <i>et al.</i> (2000); Liscum and Briggs (1995, 1996)
<i>rpt2</i> (for root phototropism)	Signaling	Okada and Shimura (1992); Sakai <i>et al.</i> (2000)
<i>nph2</i>	Signaling	Liscum and Briggs (1995, 1996); Stowe-Evans <i>et al.</i> (1998)
<i>nph3</i> (includes JK218 and <i>rpt3</i>)	Signaling	Khurana and Poff (1989); Okada and Shimura (1992); Sakai <i>et al.</i> (2000); Liscum and Briggs (1995, 1996); Stowe-Evans <i>et al.</i> (1998)
<i>nph4</i> (includes <i>msg1</i> and <i>tir5</i>)	Auxin action	Liscum and Briggs (1995, 1996); Stowe-Evans <i>et al.</i> (1998); Watahiki and Yamamoto (1997); Ruegger <i>et al.</i> (1997)

^a*rpt1* (for root phototropism) and JK224 (JK are initials of the author) are allelic to *nph1*; *rpt3* and JK218 are allelic to *nph3*; *msg1* (for massugu 1), identified in a screen for lack of hypocotyl bending in response to a unilateral application of auxin, and *tir5*, one of the auxin transport inhibitor response mutants (others, *tir1* and *tir3*, have been referred to in Chapters 13 and 22, respectively), are both allelic to *nph4*.

are potential photoreceptor mutants (Fig. 27-7B). The shift in the threshold for the fluence–response curve, it is argued, reflects a change in the properties or amount of the photoreceptor, not in signaling. In contrast, seedlings that give an impaired response at a normal fluence are potential mutants in the signaling pathway. Data from an actual screening experiment are shown in Fig. 27-7C. As can be seen, the fluence threshold, as well as the optimal curvature for the first positive phototropism, is shifted to a much higher fluence. It should also be noted that in this mutant, the fluence threshold for the second positive phototropism is little affected.

Since auxin is involved in differential growth during photo- or gravitropic bending, another screen is to apply IAA in lanolin paste on one side of the hypocotyl and isolate mutants that show a lack of curvature.

Using these screens, a number of mutants defective in the phototropic response in *Arabidopsis* have been obtained (Table 27-1). Interestingly, several mutants identified using the hypocotyl turned out to be allelic to those identified using the root in the screen, which suggests that the phototropic response occurs in the same manner in aboveground parts and in the root; although auxin thresholds may be different and different signaling components may operate in the two

systems. Another interesting feature is that some mutants, such as *nph1* and *nph3*, appear to be involved exclusively in phototropism. Despite intensive efforts to identify other developmental or morphogenic effects of these mutations, only phototropism has been identified. Other mutants, such as *nph4*, in contrast, have pleiotropic effects.

Among mutant loci, *NPH1* is thought to encode the receptor for phototropism at low fluences of blue light. A second blue light photoreceptor has been shown to mediate the phototropic response at high fluences of blue light. In the following, evidence for *NPH1* being a photoreceptor is presented first.

4. RECEPTOR FOR PHOTOTROPISM AT LOW FLUENCE IS A KINASE

Earlier studies in Winslow Briggs's laboratory, Carnegie Institute of Washington, Stanford, California, had established that etiolated pea seedlings irradiated with blue light showed rapid phosphorylation of a protein associated with plasma membrane fractions. Also, microsomal fractions extracted from dark-grown pea seedlings preirradiated with blue light and incubated with ³²P-labeled ATP *in vitro* showed

a higher fluence to reach it are potential photoreceptor mutants (mutant 1). Those that give a reduced or impaired response without a shift in fluence range are potential signaling mutants (mutant 2). (C) Fluence–response relationship for phototropism for wild-type and JK224 mutant in *Arabidopsis*. A single pulse of blue light (450 nm) was given at a fluence rate, which is indicated in the upper right corner ($\mu\text{mol m}^{-2}\text{s}^{-1}$). The curvature was measured 2 h after the beginning of the light stimulus. In this mutant, the threshold for the first positive curvature and the fluence for optimal response for this curvature are about 20–30 times higher than in the wild-type parent. Note also that although the response threshold for the first positive curvature is shifted to a higher fluence in the mutant, there is a tendency to attenuate the zone of indifference such that the fluence requirement for the second positive curvature appears not to be much affected. Vertical bars ± 1 SE; $n = 45$ –55 for each point. (Modified from Khurana and Poff (1989).

strong incorporation of the label. The phosphorylation response was subsequently observed in plasma membrane fractions from many other species (e.g., sunflower, tomato, *Arabidopsis*, zucchini, wheat, barley, oat, maize). The apparent M_r of the phosphorylated protein in different plants ranged between 114 and 130 kDa, with an approximate value of ~ 120 kDa. The protein is phosphorylated at multiple serine and threonine residues. As shown later, it also carries the catalytic domain of a serine/threonine protein kinase. Thus, the 120-kDa protein is a kinase also capable of autophosphorylation using ATP. Light apparently activates the kinase function rather than exposing sites for autophosphorylation.

Several lines of evidence indicate that the 120-kDa protein has a role in the phototropic response. (i) It is abundant in tissues that are photosensitive, and (ii) the action spectrum for phosphorylation matches that for phototropism. (iii) The kinetics of phosphorylation correlate with the kinetics of the phototropic response. This means that phosphorylation precedes evident signs of curvature and shows the same dark recovery period as phototropism following a saturating light pulse. Also, phosphorylation and first positive phototropism obey the reciprocity law.

(iv) Finally, in a study using oat coleoptiles, it was shown that unilateral blue light irradiation generates a directional gradient of protein phosphorylation. The protein, associated with plasma membrane fractions, is phosphorylated first on the irradiated side at the coleoptile tip. With increasing fluence, phosphorylation extends across to the shaded side and basipetally down the length of the coleoptile (Fig. 27-8). This pattern of phosphorylation correlates with the pattern of the phototropic curvature.

The isolation of *Arabidopsis* phototropic mutants was a signal step in confirmation that the 120-kDa phosphorylated protein is a blue light receptor. Among the mutants, the *nph1* mutant lacks the 120-kDa protein, as well as phosphorylation activity, whereas *nph2*, *nph3*, and *nph4* have normal amounts of the protein and phosphorylation activity. The mutant *rpt2* also shows normal phosphorylation activity. These data clearly suggest that the *NPH1* locus encodes the receptor, whereas *NPH2*, *NPH3*, *NPH4*, and *RPT2* participate in signaling activity. The *NPH1* gene was cloned using a map-based strategy. It encodes a protein with some interesting features.

4.1. Structure of NPH1

The deduced amino acid sequence of NPH1 indicates that the N-terminal region carries two similar,

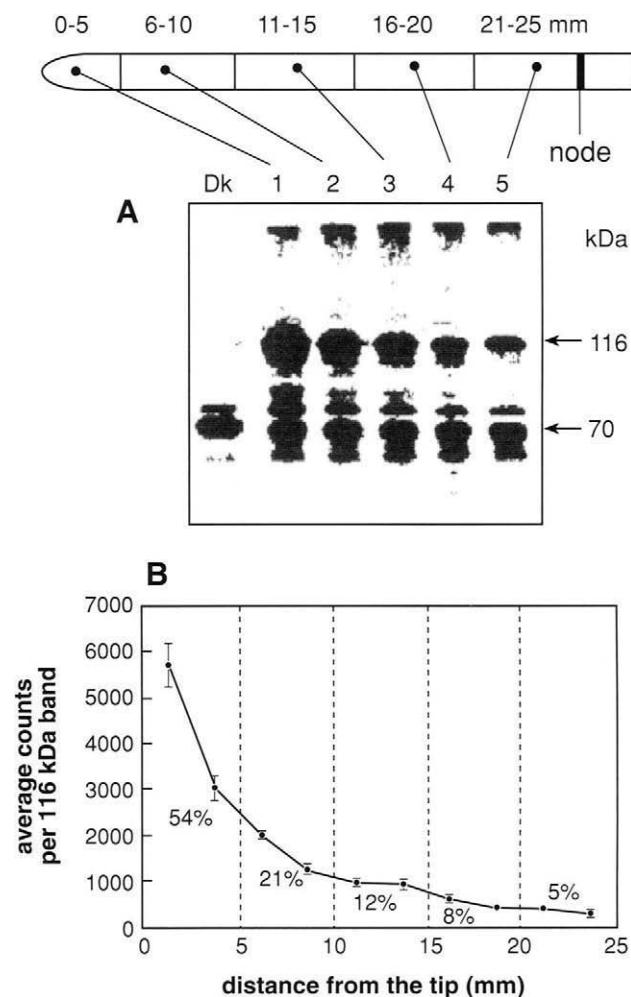


FIGURE 27-8 Blue light-dependent protein phosphorylation in an oat coleoptile. (A) *In vitro* phosphorylations of cell extracts using [32 P]ATP. Extracts were prepared from 5-mm segments of 25-mm-long coleoptiles as shown in the schematic above. Oat has a 116-kDa protein, which shows a clear decline in radiolabel from the coleoptile tip to base. In contrast, the 70-kDa protein, an internal control, shows no change in labeling. (B) The exponential basipetal decline in phosphorylation of the 116-kDa protein and the percentage of the total 116-kDa protein that is phosphorylated in each segment from the coleoptile tip to base. Each data point represents the mean \pm SD of six individual experiments. From Salomon *et al.* (1997).

but not identical, domains, designated LOV1 and LOV2, whereas the C-terminal region carries the catalytic domain of a protein kinase (Fig. 27-9A). LOV (for light, oxygen, or voltage) domains are found in a variety of sensor proteins from bacteria and eukaryotes, some of which are known to alter their redox status in response to light, oxygen, or electric voltage (hence, the acronym LOV). Some of these proteins bind flavin adenine dinucleotide (FAD) as the chromophore in their N termini. LOV domains bear similarity to PAS domains seen in many proteins, including the

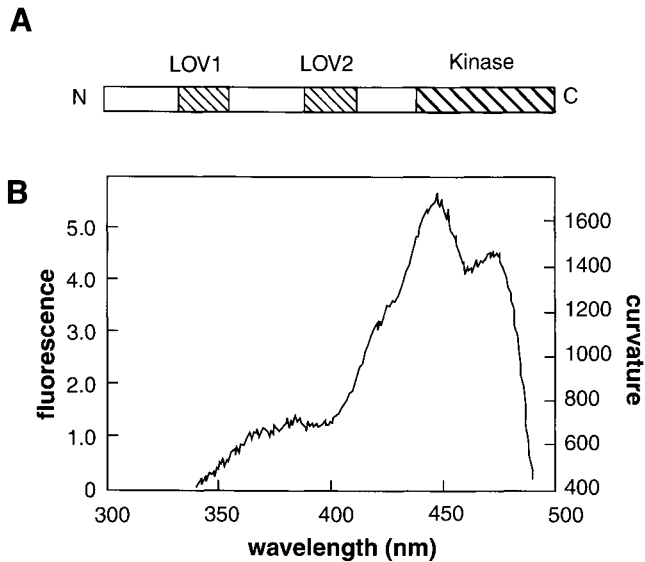


FIGURE 27-9 Structure of NPH1 protein in *Arabidopsis* and the fluorescence spectrum of NPH1 expressed in insect cells. (A) The N-terminal region carries LOV1 and LOV2 domains, both about 110 amino acids long. Each LOV domain binds a flavin mononucleotide (FMN) molecule, which serves as the chromophore. The C-terminal region carries the catalytic domain of a serine/threonine protein kinase. (B) Fluorescence spectrum of recombinant NPH1. From Christie *et al.* (1998) with permission.

phytochromes (see Fig. 26-27); indeed, LOV domain proteins are thought to constitute a subgroup of the superfamily of PAS domain-containing proteins. The C-terminal kinase domain shows all the conserved sequences, I–XI, of a protein kinase (for conserved domains in the catalytic domain of a protein kinase, see Fig. 25-9, Chapter 25) and is further identified as a serine/threonine kinase. Although the 120-kDa protein is associated with plasma membrane fractions, NPH1 shows no hydrophobic sequences or transmembrane domains. It may, therefore, be anchored to the plasma membrane *via* some lipid (or prenyl) moiety.

Expression of NPH1 cDNA in insect cells yields a recombinant protein that shows *in vitro* autophosphorylation with radiolabeled ATP. It also binds flavin mononucleotide (FMN) noncovalently, and the holoprotein so produced shows excitation fluorescence spectrum, which matches the action spectrum of the first positive phototropic curvature in alfalfa (Fig. 27-9B, cf. with Fig. 27-2A). Thus, the case for the NPH1 protein being the photoreceptor for phototropism is very strong, and the protein has been rechristened “phototropin.”

Subsequent *in vitro* studies using expressed recombinant peptides in *Escherichia coli* have shown that each LOV domain in NPH1 binds a molecule of FMN at a conserved cysteine residue. Furthermore, such binding is initiated on irradiation with a blue light

pulse and is disaggregated in the dark, thus representing the on/off steps of a photocycle. Cystein–FMN adduct formation and autophosphorylation of phototropin on irradiation with blue light activate the kinase function and probably represent the first events in phototropic signaling.

4.2. NPH1 Homologues and Similar Genes

cDNAs encoding proteins homologous to NPH1 have been cloned in oat and maize, and partial sequences are known from several other plants [e.g., pea (*Pisum sativum*), ice plant (*Mesembryanthemum crystallinum*), and spinach (*Spinacea oleracea*)]. A gene in *Arabidopsis*, called NPL1 (for NPH1-like) encodes a smaller protein, but contains the two LOV domains as well as the kinase domain; several other NPL genes have also been found in *Arabidopsis*. Two genes from rice (*Oryza sativa*) have also been cloned. While OsNPH1a is homologous to NPH1, OsNPH1b shows sequence similarity to NPL1. Thus, NPH1 and similar proteins are ubiquitous in plants.

5. HIGH IRRADIANCE PHOTOTROPIC RESPONSES ARE MEDIATED VIA NPL1

During isolation of *nph1* (allelic JK224) there were indications that the NPH1 locus might not be involved directly in the phototropic response at high irradiances. For instance, the fluence threshold for the first positive phototropism was shifted to a higher fluence, but that for the second positive phototropism was little affected (see Fig. 27-7C). However, the isolation and characterization of root phototropic (*rpt*) mutants in *Arabidopsis* provided the first clear indication that the high irradiance response might be mediated via some blue light receptor other than NPH1. Figure 27-10A shows the second positive phototropic response in wild-type *Arabidopsis* seedlings as well as in the *nph1* mutant. The wild type shows phototropic curvature at the lowest fluence rate used; the curvature increases with increasing fluence, reaching saturation at $\sim 10 \mu\text{mol m}^{-2} \text{s}^{-1}$. The attenuation of the response at $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ might be a case of adaptation at the response level. The *nph1* mutant, as expected, shows little curvature at low fluence rates, but shows a dramatic increase in curvature between 1 and $10 \mu\text{mol m}^{-2} \text{s}^{-1}$, and the curvature continues to increase and is not saturated even at $100 \mu\text{mol m}^{-2} \text{s}^{-1}$.

In the search for the second photoreceptor, several studies have shown that NPL1 encodes the high

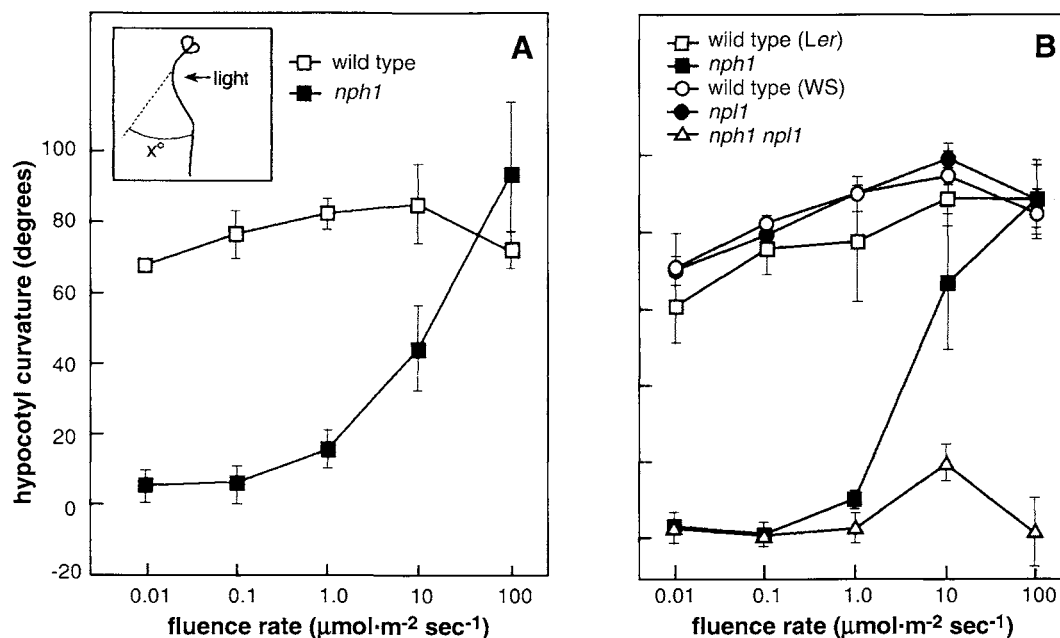


FIGURE 27-10 Second positive phototropism in etiolated *Arabidopsis* seedlings. Phototropic curvatures for the wild-type and *nph1* mutant (A); and for the wild types, *nph1* and *npl1* single mutants and *nph1 npl1* double mutant (B). The *nph1* mutant used in these experiments is actually *nph1-101*, so designated because it was isolated in a different laboratory. This mutant is in the Landsberg *erecta* (Ler) background. The *npl1* mutant is in the Wassilewskija (WS) background. Note that the *nph1* mutant shows little phototropic curvature at low fluence rates, but shows normal response at high fluence rates ($1.0\text{--}100\ \mu\text{mol m}^{-2}\text{ s}^{-1}$). The *npl1* mutant, by contrast, shows normal phototropic response at both low and high fluence rates, but the double mutant *nph1 npl1* is impaired in phototropic response at both low and high fluence rates of blue light. The normal response of *npl1* single mutant at high fluence rates may be due to functional redundancy with phytochromes and/or cryptochromes. In each experiment, the wild-type and mutant seedlings were irradiated with blue light for 12 h at indicated fluence rates before curvature measurement. Experiments were repeated three times with 8 to 16 hypocotyls measured for each data point. Data and error bars represent the mean \pm SD from average values for the three experiments. Modified with permission from Sakai *et al.* (2000, 2001).

intensity blue light receptor. In higher plants, chloroplasts in leaf mesophyll show movements which are regulated by blue light, and depending on light intensity are either an accumulation or an avoidance response. Under dim light, chloroplasts accumulate along the periclinal walls maximizing light interception for photosynthesis, but under strong light chloroplasts move from the periclinal to the anticlinal walls of mesophyll cells, leading to maximum mutual shading and protection against damage by high intensity light. It was shown recently that the *npl1* mutant in *Arabidopsis*, is defective in the avoidance movement of chloroplasts, but it is normal in the chloroplast accumulation movement in response to low intensity blue light. It was also shown that the NPL1 protein binds FMN noncovalently; and when expressed in insect cells and irradiated with blue light, like NPH1, it undergoes light-dependent autophosphorylation. Finally, the *nph1-npl1* double mutant exhibits impaired phototropic response under both low light and high light intensity blue light (Fig. 27-10B). These results clearly demonstrate that *npl1* is the

photoreceptor for the high irradiance phototropic response as well as the avoidance response by mesophyll chloroplasts.

Since a number of phototropin-like proteins are now known and more are being discovered each year, a new system of nomenclature has been adopted (see Briggs *et al.*, 2001). Based on sequence homology and likely functional differences, the angiosperm proteins fall into two major groups which have been named phot1 and phot2. The sequences in the phot1 group include *Arabidopsis* NPH1, rice OsNPH1a, maize NPH1, oat NPH1-1 and NP1-2, and pea PsPk4. The phot2 sequences include *Arabidopsis* NPL1 and rice OsNPH1b. The phot1 group is thought to be involved in low fluence blue light responses, while the phot2 group is thought to mediate the high intensity blue light responses.

Blue and red light receptors in cryptogams provide some conventional and novel combinations in the design of photoreceptors. A brief account of these receptors is provided in Box 27-1.

BOX 27-1 PHOTORECEPTORS IN ALGAE, MOSSES, AND FERNS

AS IN SEED PLANTS, many light-regulated responses in ferns, mosses, and algae are mediated by blue light receptors and phytochromes. These responses are distinguished into two physiological types. One type, a dichroic response, depends on orientation of the electrical vector of light and requires a specific orientation of photoreceptor molecules. It involves perception not only of the quality but also the direction of impinging light. The other type, a nondichroic response, is independent of the electrical vector of light. Among the former are chloroplast movement and phototropic response in algae, mosses, and fern gametophytes. Nondichroic responses include spore germination, tip growth, and cell division in ferns.

Several different blue light receptors and phytochromes occur in these groups of plants, and the existence of two physiologically distinct types of photoreceptors, one mediating dichroic the other nondichroic responses, is predicted. In addition, several responses are regulated by both blue and red/far-red light receptors, thus imparting a great variety and complexity to light-mediated responses in these plants. Until recently, it was difficult to study the light receptors in cryptogams, partly because materials were available only in small amounts and partly because these plants synthesize chlorophyll in darkness, thus making extraction and purification of phytochromes difficult. With the availability of molecular and genetic techniques, however, rapid progress is now being achieved.

Chloroplast reorientation in response to light in most plants (including angiosperms) is a blue light-mediated response. In several cryptogams, however, including fern protonemata, mosses, and green algae, it is also mediated by red/far-red light. *Mougeotia scalaris* (a filamentous green alga) shows extreme sensitivity to the red light signal and hence has been investigated intensively. In this alga, each cell has a single ribbon-shaped chloroplast, which can be reoriented in response to a single pulse of red light of low fluence. This movement is reversed by a pulse of far-red light given subsequently, which indicates clearly that it is a phytochrome response. In far-red light, the chloroplast is arranged with its thin edge perpendicular to the cell surface. Irradiated with red light it rotates and presents its flat face to the cell surface. Moreover, if a microbeam of plane-polarized light is used to irradiate one-half of the cell while the other half is irradiated with far-red light, the chloroplast twists; the part irradiated with red light presents its flat face, whereas the other half presents its thin edge. Such behavior predicts that the phytochrome molecules are anchored to some intracellular structure, possibly the plasma membrane, which helps rotate the chloroplasts. A gene encoding the apoprotein moiety in the phytochrome of *Mougeotia* has been cloned, but the deduced amino acid sequences show no transmembrane domain. The carboxy terminus, however, contains a motif that is thought to bind to microtubules. Thus, chloroplast movement in *Mougeotia* may be brought about by transduction of the light signal to cytoskeletal elements.

In contrast to *Mougeotia*, *Mesotaenium caldariorum* (a single-celled green alga), which also shows R/FR-dependent chloroplast movement, is much less sensitive to a light signal. Usually, continuous irradiation with red light or irradiation with several pulses in quick succession is required for chloroplast movement. *Mesotaenium* has been shown to have a cytosolic phytochrome with no transmembrane domain. Thus, the mechanism of phytochrome-regulated chloroplast movement in these algae is still conjectural.

Phototropism by rhizoids of *Bryonia* (a marine coenocytic alga) is reported to be mediated by a blue UV-A receptor. In mosses, *Physcomitrella patens* and *Ceratodon purpureus*, however, it is regulated via phytochromes and shows red/far-red reversal. The gravitropic response in mosses appears to be modulated by blue light. The protonemal filaments of *Ceratodon* grow negatively gravitropically (upward) in darkness. This effect is reversed by blue light given simultaneously as the gravitropic stimulus.

In ferns, such as *Adiantum capillus veneris*, blue light receptors and phytochrome act cooperatively to mediate phototropism and chloroplast movement in the gametophyte. The phytochrome involved in both responses is thought to be a dichroic type, probably bound to the plasma membrane, but details are unknown.

Some other responses in ferns are also regulated by phytochrome and a blue light receptor. The phytochrome involved in these responses is the nondichroic type. Moreover, these responses are regulated antagonistically by the blue light receptor and phytochrome. For instance, spore germination in ferns is dependent on exposure to red light. It is a typical phytochrome response in that it shows R/FR reversibility. The red light promotion of spore germination is suppressed by blue light irradiation. Tip growth of the filamentous protonemata is promoted by phytochrome, but is inhibited by B light. Irradiation with B light also promotes apical swelling of protonema (see Fig. 2-44 in Chapter 2) and subsequent cell divisions, leading to two-dimensional growth of the prothallus.

Several genes (and many cDNAs) encoding the apoprotein moieties of phytochromes and blue light receptors in these plants have been cloned. Moreover, in some plants, such as *Anaemia* and *Adiantum* among ferns, *Ceratodon* in mosses, and *Mesotaenium* in algae, small families of genes encode phytochromes. For instance, *Adiantum* has three phytochrome genes, *PHY1*, *PHY2*, and *PHY3*; *Ceratodon* has two; and *Mesotaenium* has at least two, possibly many more. In other cryptogams, only one phytochrome gene has been reported (e.g., *Selaginella*, *Physcomitrella*, *Mougeotia*).

The cryptogamic phytochromes identified to date typically show the structure common to seed plant phytochromes with a chromophore-bearing region, a hinge-like region, a PAS domain, and a histidine kinase-like domain at the C terminus. Cryptogams also show an unusual type of phytochrome, which combines the structural features of phytochrome and a blue light receptor. For example, in *Adiantum*, two phytochrome genes, *PHY1* and *PHY2*, encode phytochromes with a conventional structure. The third, *PHY3*, encodes an unorthodox protein. The N-terminal of *PHY3* is very similar to the N-terminal chromophore-binding domain of *PHYA* in *Arabidopsis*, but the C-terminal is strikingly similar to *NPH1* (the blue light receptor for phototropism at low fluences of light), with both LOV domains and all the conserved domains of the catalytic region of a Ser/Thr protein kinase (Fig. 27-11). The moss, *Ceratodon*, has two phytochromes. One has the structure of a conventional phytochrome and the other is an unusual phytochrome. It has a kinase domain but with a different structure than that in *PHY3* of *Adiantum*. It also lacks the LOV domain and the blue light receptor-binding region. Other cryptogams may have still more unusual or novel combinations of photoreceptors.

Blue light receptors in cryptogams are still very much a mystery. Use of molecular techniques, however, has allowed cloning of several *CRY* genes in *Adiantum*. In this fern, five groups of *CRY* genes, members of a small gene family, have been found. The encoded proteins show structural similarities to the protein moieties of *cry1* and *cry2* in *Arabidopsis*. They probably bind a flavin chromophore, but none of them acts as a photolyase.

In summary, many responses in cryptogams are mediated *via* phytochromes and blue light receptors. Some are mediated individually by one type of receptor, others are regulated by both, some synergistically, and others antagonistically. Genes encoding the protein moieties of many of these receptors have been cloned and their structures deduced. In several plants they occur as members of small gene families. The specific roles of these phytochromes and putative blue light receptors are still unknown. They are being elucidated by the use of mutants defective in specific responses. For example, mutants in *Adiantum* have been isolated that are defective in red light-induced phototropism, but not in red light-induced spore germination or filamentous growth.

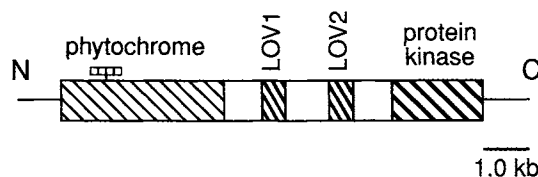


FIGURE 27-11 Structure of *PHY3* protein in *Adiantum capillus veneris*. The protein contains the domains typical of a higher plant phytochrome at its N terminus and of *NPH1* from *Arabidopsis* at its C terminus. From Nozue *et al.* (1998).

6. PHOTOTROPIC SIGNAL TRANSDUCTION

Mutant analysis to date indicates three loci that participate in phototropic signaling: *NPH2*, *NPH3*, and *RPT2*. *NPH3* and *RPT2* have been cloned. They encode similar proteins with a novel combination of two sets of domains, which are found in proteins mediating protein-protein interactions. One of these domains, a BTB/POZ (for broad complex, tramtrack, and bric-à-brac, poxvirus, zinc finger) domain, occurs near the N-terminal and the other, a coiled-coil domain, occurs near the C-terminal (Fig. 27-12).

Mutations in these two proteins do not disrupt the blue light-induced autophosphorylation of NPH1. Thus, they are thought to act downstream of primary photoreceptor signaling. The intracellular location of *RPT2* is unknown, but *NPH3* is found in the same plasma membrane fractions as NPH1 and has been shown to interact with NPH1 in yeast two-hybrid and *in vivo* coimmunoprecipitation assays. In these assays, the C-terminal coiled-coil part interacts with LOV domains. Thus, it is postulated that these proteins function as scaffold or adaptor proteins to bring together components of a signaling complex that includes phototropin (*nph1*) and/or the second phototropic receptor (*npl1*).

Substrates for phototropin acting as a kinase are unknown. *NPH3* and *RPT2* both contain multiple potential phosphorylation sites and hence could be substrates for the kinase activity. Preliminary data indicate, however, that *NPH3* is likely not a substrate for NPH1 kinase, although *RPT2* could be involved in the high fluence response.

A transient increase in free cytosolic Ca^{2+} on irradiation with unilateral blue light has been recorded in wild-type seedlings, a transient that is severely depleted in the *nph1* mutant. Thus, an early event in phototropin signaling may be mediated via Ca^{2+} /CaM-dependent or Ca^{2+} -dependent protein kinases.

7. PHOTOTROPIC RESPONSE IS MEDIATED VIA INDOLEACETIC ACID (IAA)

While the events in phototropic signaling are still very much a mystery, the ultimate response, i.e., differential growth of the stem or coleoptile, is better understood. Chapter 26 showed that the straight growth of hypocotyls/epicotyls, mesocotyls, and stem internodes is arrested by light and that both phytochromes and cryptochromes are involved in such arrest. The simplest explanation for phototropic bending is that, since light acts as an inhibitor of elongation growth, the irradiated side shows much greater growth inhibition than the opposite shaded side; hence the coleoptile/stem bends toward the light. However, the explanation is not correct. In straight growth, light inhibits growth, whereas in phototropism, there is a redistribution of growth. There is a mechanistic separation between the two phenomena, and the ecological advantages of this separation are clear. Plants can be modulated in their extension growth by light conditions, while at the same time maintaining their capacity for phototropic bending in response to a light gradient. Significantly, the mutants, *nph1* and *nph3*, affect only the phototropic response, not straight growth.

The formal concept of differential growth, the Cholodny-Went hypothesis, states that unilateral light induces a redistribution of growth, more toward the shaded side and correspondingly less toward the irradiated side. The immediate cause for this redistribution of growth is lateral migration of IAA from the irradiated to the shaded side, resulting in greater growth on the shaded side. This hypothesis, proposed in 1928, has been questioned many times, but most evidence, especially genetic evidence from *Arabidopsis* mutants, strongly supports it.

For the Cholodny-Went hypothesis to be correct, two conditions must be satisfied: (i) since curvature occurs in plant parts that are already growing,

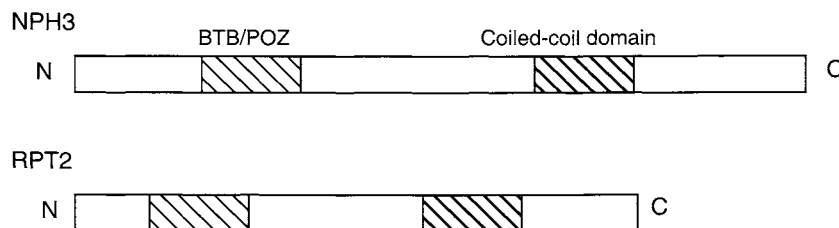


FIGURE 27-12 Structures of NPH3 and RPT2 proteins. NPH3 is a larger protein than RPT2 (predicted molecular masses of 82 and 65.8 kDa, respectively), but both have a BTB/POZ domain near the N-terminal and a coiled-coil domain near the C-terminal. From Sakai *et al.* (2000) with permission.

growth inhibition on the irradiated side should be matched by growth enhancement on the opposite side and (ii) there should be a greater concentration of IAA on the shaded side than on the irradiated side. The asymmetric distribution of IAA is not due to preferential destruction of IAA on the irradiated side because phototropism can occur at light intensities that are too low for photodestruction of IAA.

In carefully controlled experiments, it has been shown that growth inhibition on the irradiated side is indeed matched by an equal enhancement in growth on the shaded side. As shown in Fig. 27-13, the increase in growth rate on the shaded side and the decrease in growth rate on the irradiated side commenced almost simultaneously, 20–30 min after phototropic induction. The growth rate on the irradiated side almost ceased between 50 and 90 min after the phototropic induction. However, because the growth rate on the shaded side was almost doubled over the same time range, relative to the control rate, no significant change in the overall growth took place. From 90 min onward the growth rates on the shaded and the irradiated sides appeared to return gradually to the control rate.

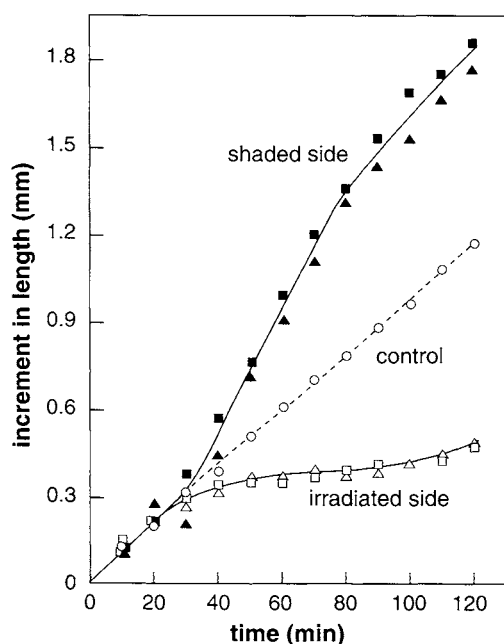


FIGURE 27-13 Time courses of growth increments on irradiated and shaded sides of maize coleoptiles. The coleoptiles were marked 15 mm below the tip with India ink, after which whole seedlings (\square , \blacksquare) or the coleoptile tip (\triangle , \blacktriangle) were irradiated unilaterally for 30 s with a pulse of blue light (fluence $5.0 \mu\text{mol m}^{-2}$). Control plants (\circ) were irradiated as for whole seedlings in a nondirectional manner. Time zero corresponds to about 3 min after the onset of phototropic induction. Each time course point is the mean obtained from 10 plants. From Iino and Briggs (1984).

A difference in the IAA concentration on the irradiated vs the shaded side of an oat or maize coleoptile tip has been shown by measurements of IAA diffusate in agar blocks, with or without prior feeding of radiolabeled IAA (Fig. 27-14). IAA concentration ratios on the irradiated vs the shaded side typically range between 1:2 and 1:3, which match the differences in growth rate on the two sides. Similar data have been reported for etiolated and de-etiolated dicot seedlings (e.g., radish hypocotyls, bean internodes).

The most convincing proof that IAA is involved in phototropic and gravitropic curvature, and possibly in all instances of asymmetric growth in axial organs, is provided by *Arabidopsis* mutants isolated in screens for auxin transport or sensitivity (e.g., *aux1*, *axr1*, *axr2*, see Chapter 22), ethylene response (e.g., *hls1*, see Chapters 15 and 21), or agravitropism in shoots or roots (e.g., *pin1*, *eir1*, see Chapter 13). All of these mutants are defective in photo-and/or gravitropism, and their wild-type genes encode proteins that have a function or presumed function in auxin transport or signaling. Also, as mentioned earlier, *msg1* mutants were isolated in a screen that utilized the IAA-dependent curvature of *Arabidopsis* hypocotyls (see Table 27-1).

In shoots, IAA synthesized at shoot tips is translocated basipetally in a polar fashion and, as shown in Chapter 13, this polar transport is mediated by auxin efflux carriers. Under phototropic stimulus by blue light, the redistribution of auxin must involve changes in activities/distribution of efflux carriers, but such redistribution still needs to be demonstrated. Since similar changes occur during gravitropism and have been better studied for that response, these changes in auxin redistribution, as well as the mechanism by which IAA brings about asymmetric growth in roots and shoots, are covered in Sections II and III.

8. PHOTOTROPISM IN GREEN (DE-ETIOLATED) SHOOTS

Despite the fact that most phototropic bending in nature occurs in green shoots, there is little conclusive data using green materials because there are several complicating factors. The fluence threshold for the first positive curvature in green tissues, using monochromatic blue light, is increased from the VLF to the LF range. This may be due in part to the masking of blue light absorption by chlorophylls and carotenoids, which also absorb in blue. A differential distribution of IAA on the shaded vs the irradiated side has also been difficult to demonstrate. Furthermore, in light, inhibitors of growth, e.g., xanthoxin, increase in

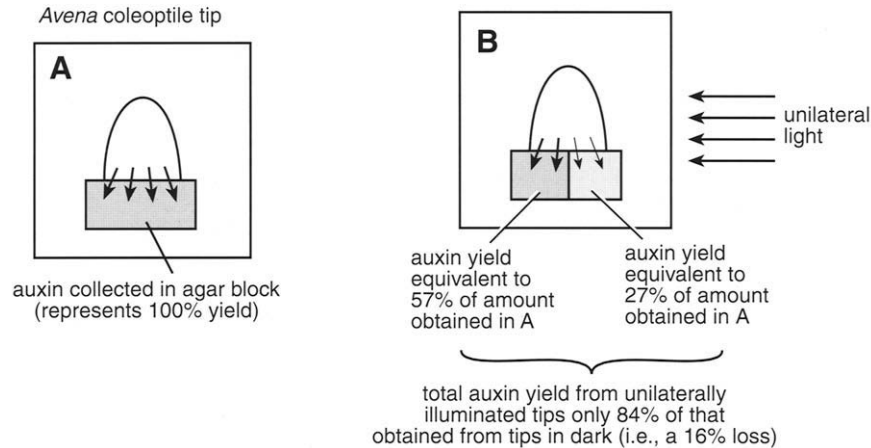


FIGURE 27-14 IAA content in *Avena* coleoptile tips before and after irradiation with unilateral light. The total auxin yield from the illuminated tip (B) was 16% less than that from tips maintained in darkness (A). This apparent loss was probably not significant. From Wareing and Phillips (1981).

concentration; if such an increase occurs on the illuminated side, this may result in curvature. High irradiance blue light may itself inhibit growth on the irradiated side of the stem causing curvature. Finally, light-grown materials show much reduced rates of elongation growth in comparison to dark-grown materials, and measurement of the phototropic curvature against that background would be difficult. Use of genetic and molecular techniques, however, is likely to provide clearer answers in the future.

9. SECTION SUMMARY

The phototropic response is mediated by blue light in a complex manner. As revealed from work with etiolated seedlings, the response typically occurs in two stages. The fluence–response curve for the first phototropism is a bell-shaped curve, which follows the law of reciprocity, whereas the second positive phototropism is a high irradiance response. The response involves at least two blue light receptors: one acting primarily at low fluences and the other primarily at high fluences of blue light. The phytochromes, though not directly involved, enhance the magnitude of the first phototropic response in a still unknown manner. In stems/coleoptiles, irradiation with unilateral light results in a gradient of blue light from the irradiated to the shaded side, which is paralleled by autophosphorylation of an ~ 120 kDa protein, which occurs ubiquitously in plants. An *Arabidopsis* mutant, *nph1*, lacking this protein shows reduced sensitivity to low fluence blue light and lacks the phosphorylation activity. The gene *NPH1* encodes a protein, called

phototropin, which is the receptor at low fluences of blue light and is thought to signal *via* a phosphorelay. The N terminal of NPH1 has two LOV domains, typically seen in proteins which sense changes in light, oxygen, or voltage in a wide variety of organisms, and a C terminal catalytic domain of a Ser/Thr protein kinase. Each LOV domain binds a molecule of flavin mononucleotide (FMN), which is the chromophore responsible for blue light absorbance. On irradiation with blue light, FMN is bound at a conserved cysteine residue, the protein is autophosphorylated, and kinase function is activated. Another gene in *Arabidopsis*, *NPL1*, encodes the protein for the high intensity blue light receptor. The structure of NPL1 is similar to that of NPH1; it also has LOV domains and a kinase domain, binds FMN noncovalently, is autophosphorylated on exposure to blue light, and is thought to signal *via* a phosphorelay. Proteins similar to NPH1 and NPL1 are known to occur in a wide variety of monocots and dicots and several homologous or similar DNA sequences have been cloned. As a result, a phototropin family of receptors, similar to phytochrome and cryptochrome families, is now recognized with two major groups in angiosperms. The groups called phot1 and phot2, are represented by *nph1* and *npl1* and are thought to mediate the low fluence and high fluence blue light responses, respectively.

The steps in signal transduction from a phosphorylated *nph1* or *npl1* are unknown and no substrates for either kinase have been discovered as yet. Among the mutant loci involved in signaling, *NPH3* and *RPT2* encode similar proteins, which show a combination of two motifs known to be involved in protein–protein interactions. They carry multiple phosphorylation sites, but they do not seem to be the direct substrates

of NPH1 kinase activity. However, they could mediate the assembly of multimeric protein complexes, which include photoreceptors as well as downstream signaling intermediates.

A phototropic curvature is brought about by a decrease in the growth rate on the irradiated side and a compensatory increase in the growth rate on the shaded side. After the curvature has been effected, the rates on the two sides return to normal. This redistribution of growth is thought to be mediated by

changes in IAA content—a decrease on the irradiated and an increase on the shaded side. That IAA is involved in the phototropic response is supported by measurements of IAA content on the two sides and from mutants that are defective in IAA uptake, transport, and/or sensitivity. How IAA may bring about asymmetric growth, the role of NPH4 protein and other genes in this growth, and the involvement of other hormones, especially ethylene, in asymmetric growth are covered in Section III.

BOX 27-2 NASTIC MOVEMENTS AND SOLAR TRACKING

THE DIURNAL MOVEMENTS IN compound leaves of some members of Fabaceae (e.g., *Mimosa pudica*, *Samanea saman*, *Albizia julibrissin*) have been well studied. Changes in the leaf (or leaflet) angle that occur at sunset or sunrise are fully and repeatedly reversible. They operate by expansion/contraction of motor cells in a special organ, pulvinus, that occurs at the base of the petiole or individual leaflets. The pulvinar epidermis often shows transverse folds, which are closer together and deeper along the contracting flank and wider spaced and shallower along the expanding flank. The central vascular core is surrounded by a massive sheath of motor cells. Because the changes in leaf angle occur along one plane, two regions of motor cells on opposite sides undergo expansion/contraction or reversible turgor and size changes. Cells whose turgor increases during leaflet opening and decreases during closure are called “extensors,” whereas cells that exhibit the reverse changes are called “flexors” (Fig. 27-15). In some species, extensor cells occur on the upper side of the pulvinus and leaflets close in an upward direction (e.g., *Albizia* and *Mimosa*), whereas in others they occur on the lower side and leaflets close in a downward direction (e.g., *Samanea* and *Phaseolus*).

The expansion of extensor cells involves an influx of K^+ , Cl^- , and other ions accompanied by water uptake, and their contraction occurs because of an efflux of ions and water. Simultaneously, reverse changes occur in flexor cells. The influx of K^+ occurs via voltage-dependent K^+ channels, which are activated when the membrane potential drops below a certain level because of the activity of H^+ -ATPases in the plasma membrane and/or tonoplast. The latter in turn may be driven by the biological clock, by a light signal, or, in the case of *Mimosa*, by touch.

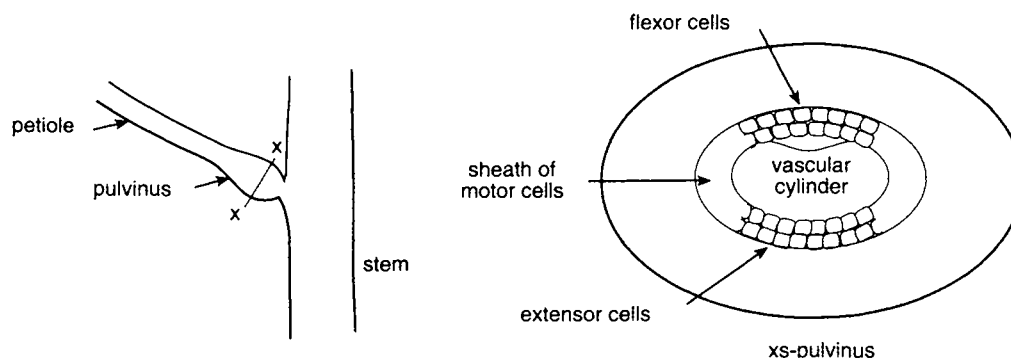


FIGURE 27-15 Schematic drawings showing the location and structure of a leaf pulvinus. Pulvinus occurs as a swollen structure at the base of the leaf petiole (left). A thick sheath of motor cells occurs in the cortex external to the vascular cylinder (right). Motor cells on one side act as extensor cells and on the opposite side as flexor cells.

Solar tracking is a directional response to light, but it differs from phototropism in that plant organs track the light source, not merely turn toward a fixed light source. Also, it usually involves mature tissues. Solar tracking by leaves, leaf heliotropism, is known in about 16 families of angiosperms; it is thought to maximize light interception under shady conditions or short growing seasons. Solar tracking by flowers is known from four families: Asteraceae, Papaveraceae, Ranunculaceae, and Rosaceae. In these families, it is usually arctic or alpine species that show floral heliotropism. Heliotropism raises flower temperature, enticing insects to remain, basking and foraging, enhancing their effectiveness as pollinators. In the snow buttercup (*Ranunculus adoneus*), a high alpine perennial, solar tracking raises temperatures within the flower bowl to up to 3.8°C above the ambient. Foraging insects spend more time in well-tracking flowers than in poorly tracking flowers, which produce fewer seeds.

Leaf heliotropism has been studied in members of Fabaceae and Malvaceae, where it is believed to result from short-term fluctuations in turgor pressure in motor cells of the pulvinus at the base of the petiole. In this case, however, the acquisition and release of turgor of motor cells probably occur in a wave around the circumference of the motor cell sheath. In contrast, floral heliotropism in the snow buttercup is reported to involve differential cell growth, more toward the shaded side than toward the irradiated side.

Many of the aforementioned responses, including stomatal pore opening, all known cases of solar tracking, whether by leaves or flowers, and the photonastic unfolding of leaflets in *Mimosa*, *Albizia*, and *Samanea*, are triggered by blue light, and action spectra show the typical three-finger pattern as in phototropism. The nyctinastic folding of leaflets in Fabaceae, however, is promoted by red light, if given close to the end of the day. This red light-induced closing is reversible by far-red light, hence it is mediated by phytochrome. How two distinct photosystems bring about opposite responses in nastic folding and unfolding of leaflets is not clear. Since this response shows a diurnal periodicity and is subject to endogenous signals generated by the biological clock and since both blue and red lights are known to be involved in resetting the clock, it may be that these effects are mediated via the clock.

For stomatal pore opening, there is considerable evidence that a carotenoid, zeaxanthin, is the chromophore for the blue light receptor. However, for the other responses, the photoreceptor(s), the details of signal perception and the mechanics of organ movement are poorly understood.

SECTION II. GRAVITY-RELATED PHENOMENA

Gravity is the most constant and pervasive of all environmental signals. In an aqueous habitat, because of buoyancy, gravity hardly affects the development of the organic form, but in a terrestrial habitat, organisms have to contend with 1g gravity throughout their lives. Gravity-directed growth processes, called gravitropism (in earlier literature, it was referred to as "geotropism"), affect both shoots and roots. Roots are considered positively gravitropic and stems are considered negatively gravitropic, but these are the broadest of generalizations. Roots and shoots always perceive gravity, but they may not display perfect positive or negative gravitropism. In fact, they show all the angles from 0 to 180° on the gravitational axis, where 0 is straight down and 180 is straight up (Fig. 27-16). Orientation with respect to the axis of gravity also changes developmentally. For instance, only the larger or primary roots grow in parallel with the gravitational axis; roots of the second, third, and fourth order grow at various angles all the way to 90° to the

gravitational axis (or horizontally). Thus, a system of nomenclature has been built up: axes that align themselves parallel to the direction of earth's gravity are called *orthogravitropic*, e.g., most main roots and shoots; axes that align themselves at right angles to the direction of earth's gravity are *diagravitropic*, e.g., rhizomes and stolons; axes that align themselves at angles between 0 and 90° are *plagigravitropic*, e.g., most branches and lateral roots of the first order; and axes that are insensitive to gravity are *agravitropic*, e.g., lateral roots and branches of second and third order, aerial roots of orchids, and some other epiphytes.

The gravitropic response has been studied mostly in vertically growing (orthogravitropic) systems, such as seedling roots or stems/coleoptiles of model plants such as maize, tomato, mung bean, tobacco, and, in more recent years, *Arabidopsis*. Our information on gravity sensing in systems growing at some angle from vertical (plagigravitropic) and naturally agravitropic roots and shoots is almost nonexistent. This section deals mainly with the gravitropic response in orthogravitropic roots and shoots—how gravity is

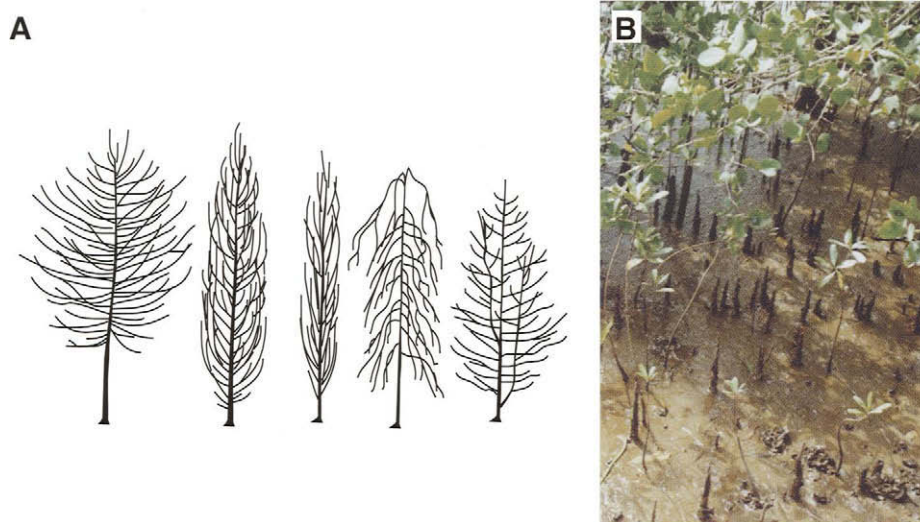


FIGURE 27-16 Branching patterns in shoots and some atypical roots. (A) Branching patterns and architecture of some trees. From left to right, *Carpinus betulus*, *C. betulus* var. *fastigiata*, *Fagus sylvatica* var. *fastigiata*, *F. sylvatica* var. *pendula*, and *Liquidamber styraciflua*. From Finn and Digby (1997). (B) Negatively gravitropic roots, called pneumatophores, of a mangrove plant, *Rhizophora mangle*. The plant grows in swampy intertidal waters and these roots come up above the muddy surface and serve for aeration.

perceived, where it is perceived, and how plants use it to modulate their growth.

Other gravity-related phenomena include gravimorphism and gravitaxis. An example of former is the formation of reaction wood in branches of conifer and hardwood trees, possibly in response to perception of their own weight. Such reaction wood is formed on the lower side of the branch in conifers (compression wood) and on the upper side of the branch in angiosperms (tension wood). Examples of gravitaxis are common in unicellular flagellates (e.g., *Euglena*, *Chlamydomonas*) but seem to be rare, if not absent, in vascular plants.

Responses to touch or wind or rain can be grouped together with gravity perception under the heading pressure-induced responses. Touch movements are shown by climbing plants where tendrils (e.g., pea, grape vine), stem tips (e.g., bean), leaf tips, petioles, flower peduncles, and even roots (e.g., touch pads in ivy) may be used for twining around a support. Plants are extraordinarily sensitive to pressure changes: tendrils are known to respond to the weight of a cotton thread or weights as small as 20 μ g. Stroking a seedling with a piece of paper elicits inhibition of elongation and thickening of stem. Shaking a seedling even for 30 s per day is enough to inhibit elongation growth by two to six times over control. For reaction wood to form a bending of a branch by 10° is enough.

These responses can be extremely rapid, with as little as 10–30 s elapsing between stimulus and response. Special touch genes have been identified in

Arabidopsis. mRNAs of some of these genes increase in abundance 10- to 100-fold within 10–30 s of the plant being stimulated and their expression is tissue specific. For most part, however, how these signals of touch and wind are perceived is unknown.

1. GRAVITROPIC RESPONSE IN AN ORTHOGRAVITROPIC ORGAN

If a plant is placed horizontally, i.e., gravistimulated, the root curves downward toward gravity, while the stem curves upward. The curvature in both organs occurs in the subapical region, where most elongation growth occurs, and is brought about by the differential growth of cells on the physically upper vs the lower side of the organ (Fig 27-17A). After the curvature is complete, normal growth resumes, upward for stem and downward for root, whereas the site of curvature recedes basipetally into the mature zone. In cereal crops, in addition, localized and specialized areas in stems or leaves, called pulvini (singular, pulvinus), retain the capacity for growth long after neighboring cells have matured. (These pulvini are structurally and functionally different from pulvini associated with nastic movements of leaves or leaflets or solar tracking.) In Panicoid grasses (e.g., maize), the pulvinus occurs above each node but below the internode above. In Festucoid grasses (e.g., wheat, barley, oat), smaller pulvini occur at the

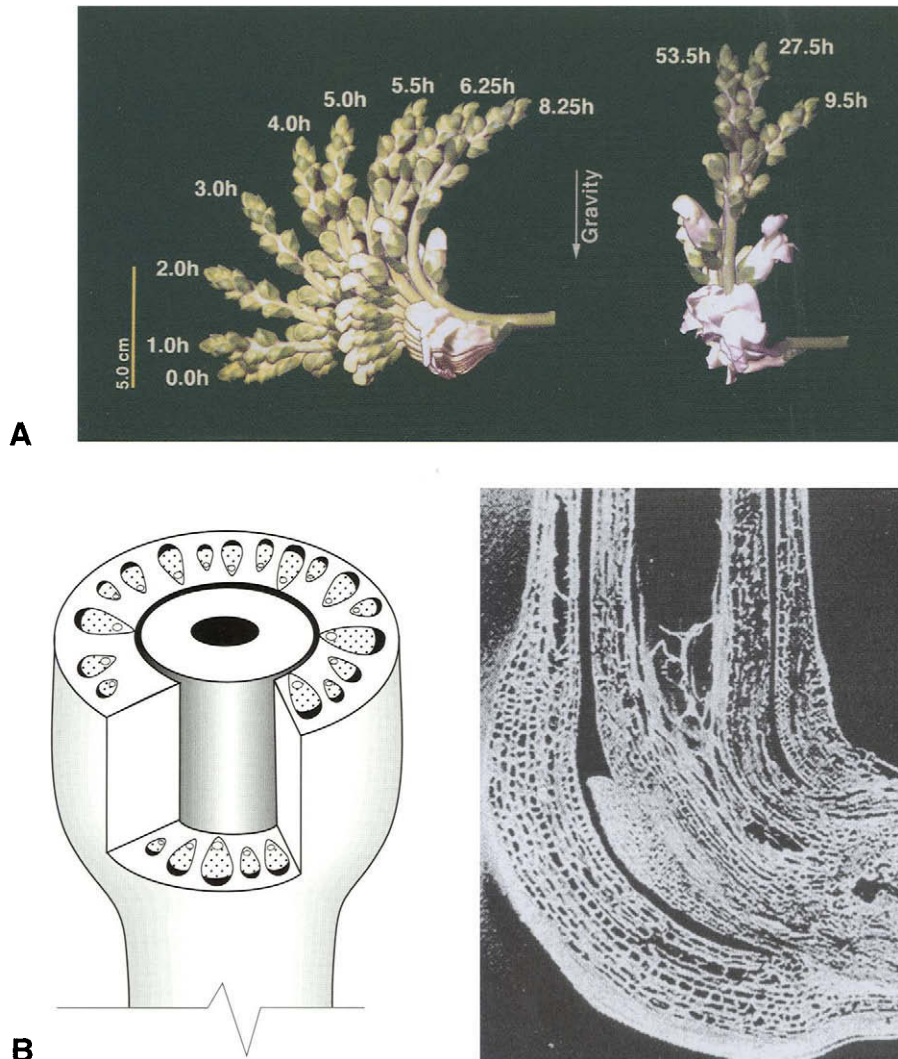


FIGURE 27-17 (A) Gravitropic response in a flowering stalk of snapdragon (*Antirrhinum majus*). The flowering stalk was placed horizontally (gravistimulated) at time 0. Photographs represent simulated time-lapse photographs. Actual photographs were taken at intervals shown on the left; they were scanned into a computer after which a time-lapse effect was created by overlapping several individual images. The photographs show upward bending of the growing part of the shoot and overbending during the first 9.5h, followed by reversion to an upright position, which is shown in the photograph on the right. After the curvature is complete, normal upward growth resumes. Courtesy of Peter Kaufman, University of Michigan, Ann Arbor, Michigan. (B) Diagrammatic representation of the leaf-sheath pulvinus region in a festucoid grass (left) and scanning electron micrograph of a gravistimulated leaf-sheath pulvinus from *Muehlenbergia schreberi* (right). In the diagram, the pulvinus surrounds the base of the internode, which appears as the central hollow cylinder. Vascular bundles (white circles) are sheathed on the abaxial side by parenchyma cells involved in graviperception (stippled) and by collenchyma tissue (black). The scanning micrograph is a longitudinal sectional view of the pulvinus (p), an axillary bud (b), and part of stem or base of the internode (s). The plant was gravistimulated for several days to exaggerate the pattern of response. Note that the cells on the outer (lower) side of the pulvinus have grown much larger than those on the inner (upper) side. From Dayanandan *et al.* (1976).

junction of the leaf sheath and the leaf blade. Should the action of wind or rain prostrate the shoots, as often happens after a rain storm, pulvinar cells show differential growth and “straighten” the stem or leaves (Fig. 27-17B).

In the laboratory, a clinostat is used for experimental work on gravitropism. A clinostat is a device used to hold a plant (or plant part) in a horizontal position. It can also be rotated at defined speeds (rotations per minute, rpms) to eliminate the effect of gravity on

growth, which allows the root or shoot to grow independently of the gravity vector. A clinostat is used to measure the basic parameters of the gravitropic response, such as the time course of curvature after horizontal placement and gravitropic sensitivity. The latter involves measurements of presentation time (defined as the minimum duration of continuous gravistimulation required for a response) and perception time (defined as the minimum time for intermittent stimulation repeated “*n*” times to evoke a response).

2. SITE OF GRAVITY PERCEPTION

The sites of graviperception differ in roots and shoots. In roots, experiments spanning more than a century have indicated that root tips are the site of graviperception and, more specifically, central cells in the columella of the root cap (Fig. 27-18A). Evidence comes from several types of experiments. Surgical experiments by Ciesielski (1872) and Darwin (1880) showed that roots of plants, such as, pea, lentil, and broad bean, would not respond to a gravitropic-stimulus if root tips were cut off until a new root tip and root cap had been regenerated. At the turn of the 20th century, centrifugation experiments in which a plant is so positioned along the radius of a centrifuge that different parts of the plant are exposed to different acceleration forces and, hence, gravitational fields, indicated that graviperception occurs within the apical 1.5 to 2.0 mm from the tip. Thus, both surgical and centrifugation experiments focused attention on the root tip and the root cap. This was followed by the demonstration in the mid-1960s that removal of the root cap from the tips of maize roots allowed continued growth in length, but inhibited a gravitropic response until the root cap was regenerated. Within the root cap, the central cells of the columella show a highly polarized structure (see later) and are thought to be the sites of perception. It has been shown that ablation of central columella cells in *Arabidopsis* roots by laser beams results in much reduced gravitropic sensing. Thus there is ample evidence from various sources that root tips, specifically central columella cells in the root cap, are the sites of graviperception. However, columella cells are not the exclusive or the only site for graviperception in roots. Many authors believe that perception extends to more proximal sites in the root, possibly as far as the cells between the meristematic zone and the main elongation zone.

In contrast, in shoots and grass pulvini, the site of graviperception is the starch-containing sheath of cells that surrounds the vascular bundles (Fig. 27-18B).

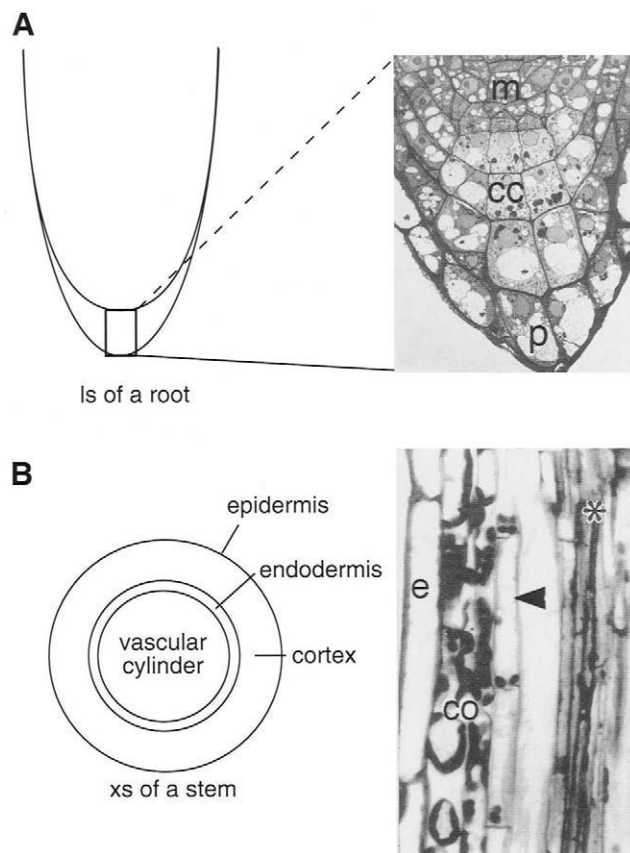


FIGURE 27-18 Sites of graviperception in roots and shoots. (A) Schematic drawing of a longitudinal section (ls) of a root with root cap (left) and a low magnification electron micrograph of the root cap region (right) showing central columella (cc) cells. These cells are derived from the root meristem (m) and, with continued meristem activity, pass progressively into the peripheral tissue (p) and eventually are sloughed off. Only central columella cells in the root cap are involved in gravisensing. Courtesy of John Kiss, Miami University, Oxford, Ohio. (B) Schematic drawing of a cross section (xs) through a stem showing the location of the starch sheath (or bundle sheath) cells. This layer, also referred to as the endodermis, occurs outside the vascular cylinder and represents the innermost layer of cortex. The same cells are shown in a longitudinal section in the accompanying light micrograph. The endodermis is indicated by an arrowhead; the vascular cells are marked with an *; co, cortex; e, epidermis. From Weise and Kiss (1999) with permission.

Developmentally, this sheath represents endodermis, or the innermost cell layer in the cortex, even though it lacks Casparian strips, typical of endodermal cells in roots. In the *scarecrow* (*scr*) and *short root* (*shr*) mutants of *Arabidopsis*, an endodermis fails to develop (see Chapter 3). The defect is seen both in roots and in shoots. If seedlings or adult plants of these mutants are subjected to gravitropic stimulus, the hypocotyls or inflorescence stems show a lack of gravitropic response because they lack an endodermis (Fig. 27-19). In contrast, roots of mutant plants show a gravitropic response because graviperception occurs in columella cells in the root cap.

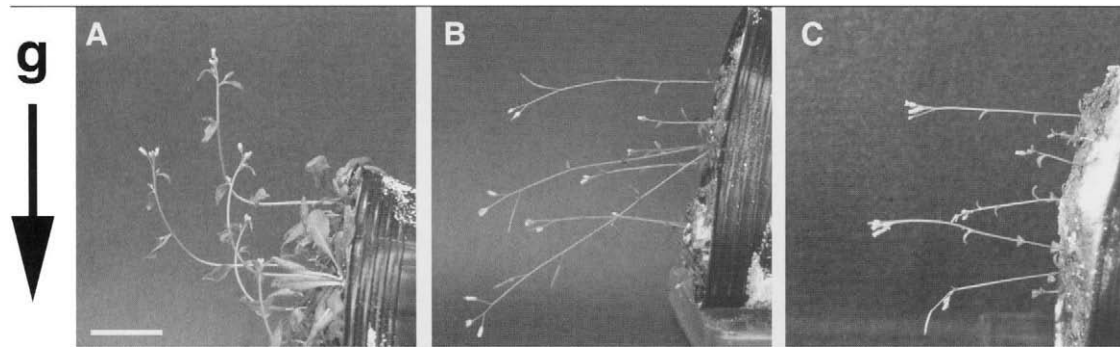


FIGURE 27-19 Gravitropic response in wild-type (A), *scr* (B), and *shr* (C) mutants of *Arabidopsis*. Only the inflorescence axes are shown. Wild-type and *scr* axes were gravistimulated for 6 h and *shr* axes for 24 h, in darkness before being photographed. Wild-type axes show upward bending, whereas *scr* and *shr* axes do not. Scale bar: 2 cm. From Fukaki *et al.* (1998).

2.1. Statoliths and Statocytes

Perception of gravity requires an object of a certain mass and a capacity to be displaced from its position of equilibrium (see Box 27-3). Sensors that

work in this way are called susceptors or statoliths. The displacement of statoliths creates a signal that is perceived by the receptors. The signal is then modulated as necessary and is transmitted to the site of response.

BOX 27-3 PERCEPTION OF GRAVITY IN PLANTS

GRAVITY IS THE ACCELERATION vector of the gravitational field of Earth and is defined as $1g = 9.81 \text{ m s}^{-2}$. The effects of gravity perception on growth are seen when the position of a plant (or root/shoot) is changed from its normal vertical orientation (dynamic stimulation); they are also seen when the root/shoot grows at a constant angle with respect to gravity (static stimulation). A striking example of static stimulation is the formation of reaction wood in stems of conifer and hardwood trees. This box deals only with dynamic stimulation.

For gravity perception (susception) in dynamic stimulation, an object must not only have a mass, it should also be capable of displacement from its equilibrium position. Also, the work done by gravity has to be higher than the elemental kinetic energy $\frac{1}{2} kT = 2 \times 10^{-21} \text{ J}$ (20°C) to discriminate between gravity-dependent movement and thermal background (e.g., Brownian movement). Assuming a mass M for an object, the work done by gravity to displace mass M during susception is mass times distance (D). Intracellular organelles are buoyed by the cytoplasm, and mass is the reduced weight of M (or m), which is equal to the volume times the difference in densities of M and the cytosol. For organelles that may be involved in susception, the following minimum condition must be fulfilled: $\Delta mgD > 2 \times 10^{-21} \text{ J}$ for $D \ll \text{cell length}$, where Δmg is the reduced weight of M ; the two vectors g and D are multiplied scalarly.

The reduced weights (mg) can be calculated for cell organelles, and knowing cell dimensions, the minimum condition can also be calculated. For example, it can be calculated that a cell $20 \mu\text{m}$ in diameter is too short to fulfill the minimum condition for mitochondria (diameter $0.5 \mu\text{m}$, density 1.2 g cm^{-3}). Plastids generally fulfill the condition, as do nuclei. However, fulfilling the minimal condition also requires that the object be free to be displaced. Plastids containing starch (amyloplasts) in guard cells, for instance, have the required mass, but are not capable of displacement. Nuclei, likewise, are held in place by a system of endomembranes and cytoskeleton and are not usually displaced. An amyloplast in a central columella cell in the root, in contrast, has the required mass and is capable of being displaced with sufficient energy to activate a receptor with a signal significantly above the background noise.

The cells harboring statoliths are referred to as statocytes. If an organism, or an organ, containing statocytes is tilted at an angle from the vertical, it causes the statolith(s) in the statocyte to be displaced from its position of equilibrium; the farther or greater its lateral displacement, the greater the signal generated. In plants cells, among all the organelles, amyloplasts, or plastids containing starch, are the only structures that have the required mass and mobility to activate a receptor with enough energy to give a meaningful signal. Amyloplasts with or without starch grains are dense and unencumbered enough (from other membranes and cytoskeletal elements) to be capable of vertical and lateral displacement in the statocyte (Fig. 27-20A). Thus they have been considered as statoliths for a long time. Presumably, starch grains released with a breakdown of plastid membranes can also serve as statoliths.

Other structures in plant cells, which can be considered as statoliths, are crystals of various kinds. Crystals of calcium oxalate, calcium carbonate, or silica are abundant in vacuoles or membrane-bound vesicles in plants. Nonetheless, amyloplasts are the most universally present structures in plant organs, such as roots and shoots, which show gravitropic responses. In fungi

and algae, however, crystals are used as statoliths. Calcium phosphate and protein crystals occur in fungi. In sporangiophores of *Phycomyces blakesleanus*, for instance, octahedral protein crystals occur in the central vacuole and serve as statoliths. Rhizoids in the green alga, *Chara*, accumulate vesicles containing crystals of barium sulfate at the tip. If the rhizoid is placed horizontally, the vesicles fall to the lower side and provide a sensing mechanism (Fig. 27-20B–D).

It has also been proposed that the entire protoplast of a cell, with all its organelles, may serve as the statolith. This hypothesis, known as the protoplast pressure model, postulates that the protoplast, which has a mass, could be displaced under a gravitropic stimulus. Such displacement would cause tension at the plasma membrane–cell wall boundary on one side and compression on the other (Fig. 27-21A). These pressure changes could be sensed via integrin-like proteins in the plasma membrane and transmitted via the cytoskeleton to the nucleus or the response site. If amyloplasts with or without starch grains are present, that would add to the mass of the protoplast. The hypothesis is supported by the observation that in the large internodal cells of some algae (e.g., *Chara*),

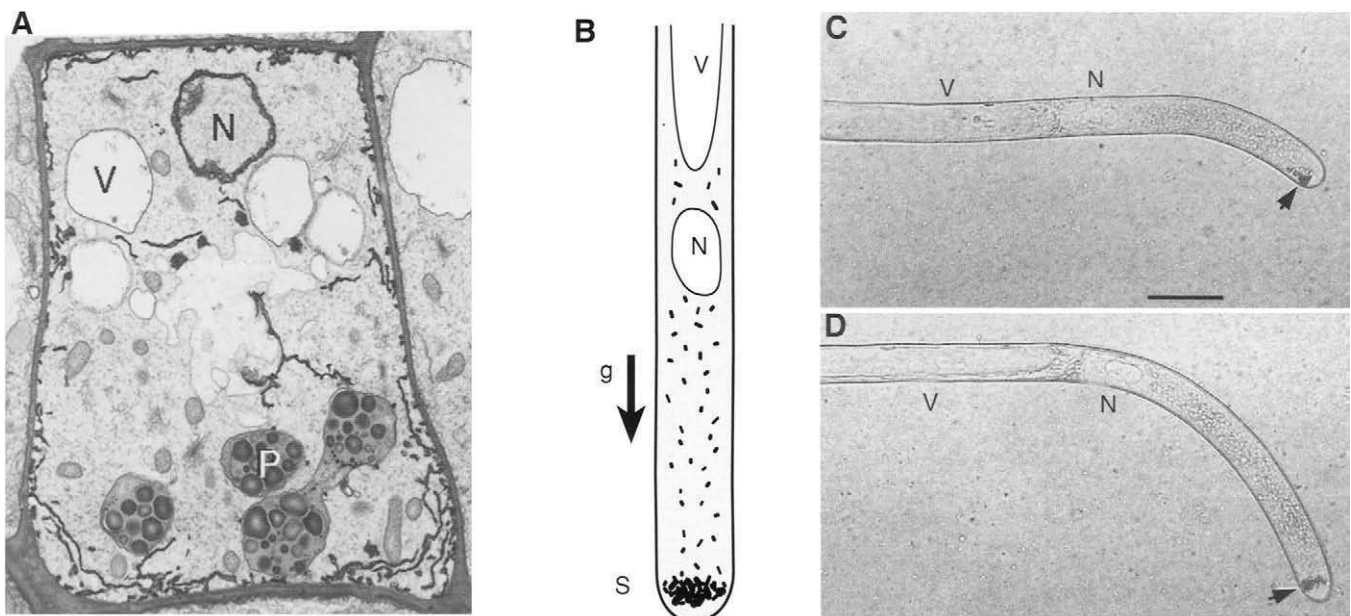


FIGURE 27-20 Two types of statoliths in plants. (A) Amyloplasts in a central columella cell of the root cap of *Arabidopsis*. These cells have a highly polarized structure, with amyloplasts (P) concentrated toward the lower basal end of the cell, whereas the nucleus (N), other organelles, and vacuoles (V) occur more toward the apical end. As the central columella cells are displaced toward the periphery, they lose their statocyte characteristics. Courtesy of John Kiss, Miami University, Oxford, Ohio. (B) Part of a growing *Chara* rhizoid in normal vertical orientation. Statolith vesicles (S) containing crystals of barium sulfate occur near, but not at, the tip. The nucleus (N) and vacuole (V) occur proximally; g, direction of gravity. Photographs on the right show graviresponse. (C) A rhizoid at 45 min after being placed horizontally. Statoliths have sedimented toward the new lower cell wall. (D) Rhizoid 3 h after reorientation. Statoliths have almost returned to their normal position found in a vertically oriented rhizoid. Bar for C and D: 40 μ m. From Kiss (2000).

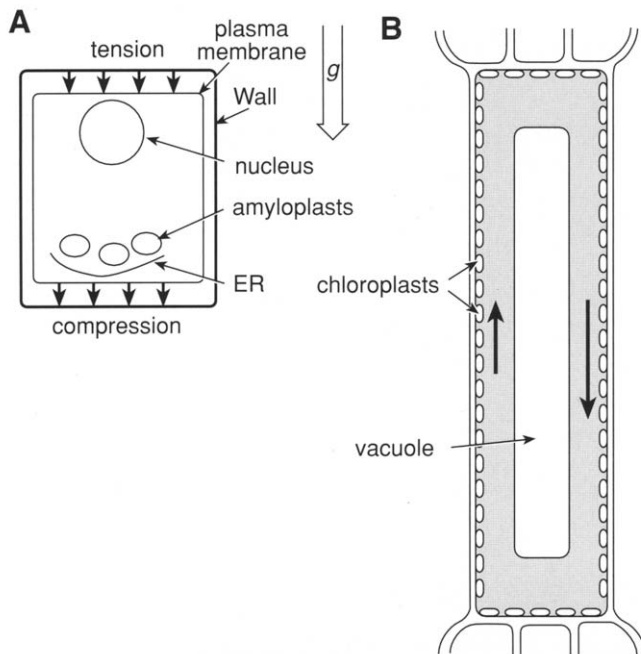


FIGURE 27-21 Protoplast pressure model and cytoplasmic streaming under gravity. (A) The mass of the entire protoplast of a root columella cell provides a signal in the form of compression at the bottom of the cell and/or tension at the top. Amyloplasts shown at the bottom provide “ballast” and add to the weight of the protoplast. From Sack (1997). (B) Schematic drawing of a young internode of characean alga. Chloroplasts are anchored in the parietal cytoplasm and are not free to move. The downward streaming in the endoplasm occurs at a faster velocity than the upward streaming (shown by different size arrows). From Weisenseel and Meyer (1997).

held vertically, cytoplasmic streaming occurs at a faster velocity in a downward direction than in the upward direction (Fig. 27-21B). This difference in velocities tends to be nullified at $10^{-4}g$ in rocket flight. Most authors believe, however, that the protoplast pressure model, while it may be applicable to the large internodal cells of *Chara* sp., which do not have any sedimenting objects, has limited applicability to root and shoot gravitropic responses in vascular plants. Nonetheless, it may contribute to a secondary gravity-sensing mechanism (see Section 2.2).

2.2. Structure of Statocytes

Light and electron microscopic studies on central columella cells in roots indicate that statocytes are highly polarized cells that contain a peripheral system of endoplasmic reticulum (ER), a nucleus positioned in the middle or at the top, and many amyloplasts toward the lower end of the cell (see Fig. 27-20A). Amyloplasts are thought to be enmeshed in a network of actin filaments (microfibrils) and suspended delicately by the filaments on a bed of asymmetrically disposed ER membranes. The ER is anchored by a

criss-crossed pattern of cytoskeletal elements, including microtubules, to the plasmalemma at the morphologically lower end of the cell. If the root is tilted from the vertical axis within a gravitational field, the amyloplasts are displaced to a new bed of ER membranes (Fig. 27-22). This displacement of amyloplasts from their equilibrium position (i.e., in vertical orientation) is perceived by the ER and plasma membranes and activates a signal transduction pathway, leading to a physiological signal, which, in turn, is responsible for the root curvature at the response site. In some cases, the movement of amyloplasts, following horizontal placement of roots, has been recorded to occur as early as 6 s after the stimulus, which is good evidence that they are involved in graviperception.

Supporting evidence for the role of amyloplasts as statoliths comes from studies utilizing the enzymatic removal of starch grains or growing plants under conditions that do not favor starch synthesis. For example, treating plants with an active gibberellin (GA) reduces the accumulation of starch. With the availability of starch-deficient mutants [e.g., in maize, tobacco (*Nicotiana sylvestris*), *Arabidopsis*], however, the emphasis has shifted to using mutants. These mutants, deficient in an enzyme in starch biosynthesis, produce less starch and yield amyloplasts that are less dense than those in the wild type (Fig. 27-23). These mutants still show gravitropism in roots and shoots, although the response kinetics are much slowed down compared to that in the wild type. In severe starchless mutants, the plastids in central columella cells may not sediment, yet the mutant roots are still capable of sensing gravity, leading to the belief that some secondary mechanism may also be involved.

Experiments using high gradient magnetic fields (HGMF) confirm that amyloplasts serve as statoliths. The HGMF can be restricted to as little as 1.0-mm

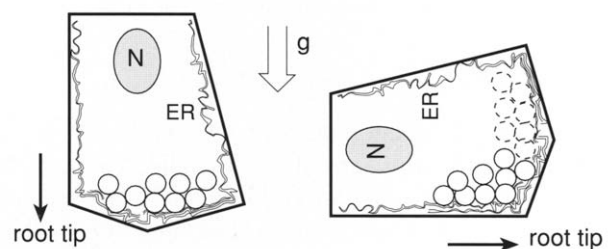


FIGURE 27-22 Mechanism of gravity sensing in root. (Left) A statocyte cell showing a nucleus toward the basipetal end, an asymmetrically disposed, peripheral ER, and amyloplasts toward the apical end (bottom). (Right) When the root is gravistimulated, amyloplasts are displaced toward the new physical bottom (original positions are shown by dashed outlines). The gravity vector and the direction of the root tip are indicated. Modified from Chen *et al.* (1999).

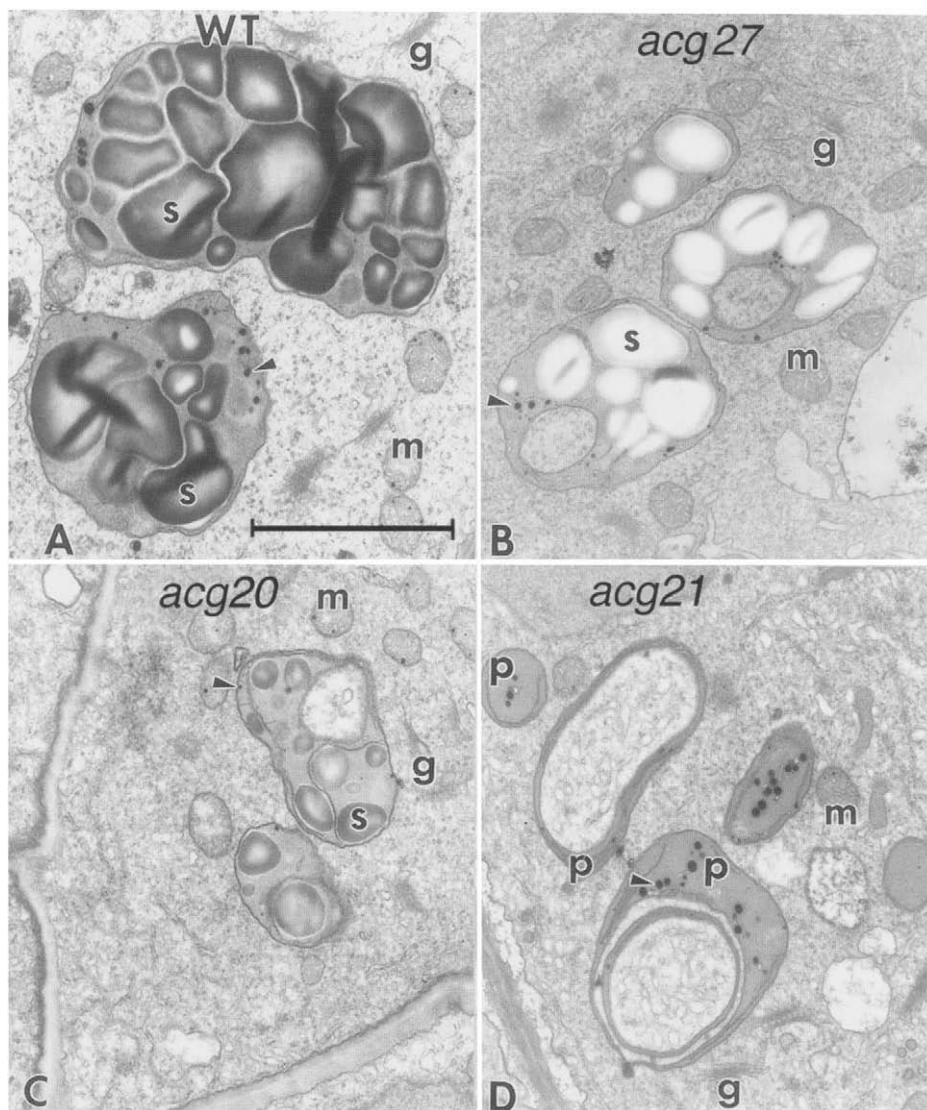


FIGURE 27-23 Electron micrographs of amyloplasts in columella cells of wild-type and starch-deficient mutants in *Arabidopsis*. (A) Wild-type amyloplasts are filled with large starch grains. (B and C) Intermediate mutants (*acg27* and *acg20*, respectively) show fewer and smaller starch grains compared to the wild type. (D) The starchless mutant (*acg21*) lacks starch. Arrowhead, plastoglobuli; g, Golgi body; m, mitochondria, p, plastid, s, starch grain. Bar: 2 μ m. From Kiss *et al.* (1996).

lengths along the plant stem or root (Fig. 27-24). It has the potential to mimic gravity in a localized area of the plant axis by displacing amyloplasts within statocytes, causing curvature to develop. Application of HGMF to *Arabidopsis* root tips has been shown to promote a lateral displacement of root cap amyloplasts and a subsequent development of curvature in the direction of statolith displacement. Similarly, in barley coleoptiles, tomato hypocotyls, and *Arabidopsis* inflorescence stems, amyloplasts displaced in starch-sheath cells cause a curvature to develop in an opposite direction

to the displacement direction, consistent with negative gravitropism in shoots.

3. GRAVITROPIC SIGNAL TRANSDUCTION

The displacement of amyloplasts on ER membranes is the first step in signal perception, but the subsequent steps of signal transduction to the site of response, despite enormous effort, are still uncertain. The

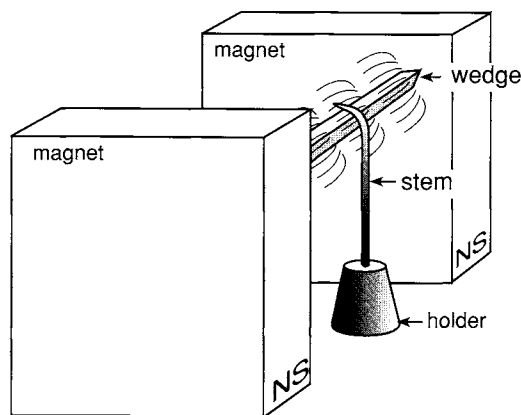


FIGURE 27-24 Diagram of the magnetic system used for the induction of curvature in inflorescence axes of *Arabidopsis*. A ferro-magnetic wedge was inserted between serially mounted magnets ($50 \times 50 \times 12.5$ mm). Wedges generated the high gradient magnetic field, as symbolized by the diverging field lines. The stem was mounted in an adjustable holder at variable angles to the wedge. The typical response (as shown) was curvature of the more apical regions of the stem toward the wedge. The entire apparatus was placed on a clinostat and rotated around an axis parallel to the center of the magnets. From Weise *et al.* (2000).

response itself, i.e., differential growth on the upper and lower sides of a horizontally placed organ, is better understood, however.

The differential pressure caused by the displacement of amyloplasts is probably perceived by the ER and/or plasma membrane. It may affect some stretch-activated channels and translate the pressure signal into an ionic or electrical signal. Ca^{2+} transients have not been observed so far in response to gravistimulation, but the possibility remains open that local transient increases in cytosolic Ca^{2+} trigger a signaling pathway. In support of this view, inhibitors of stretch-activated channels (e.g., Gd^{3+} and La^{3+}) eliminate gravitropism. Inositol-1,4,5-triphosphate (IP_3), which is a ligand for Ca^{2+} release from intracellular storage sites; Ca^{2+} -ATPases, which pump Ca^{2+} into storage sites or the apoplast; and calmodulin (CaM) and Ca^{2+} /CaM-dependent protein kinases have all been implicated. However, whether there are specific receptor proteins in the ER/plasma membranes to sense the pressure differential or the manner in which the secondary messengers translate the signal into differential growth at the response site are unknown.

Rapid changes in cytosolic pH within columella cells and in apoplastic pH of the root cap occur on gravistimulation of roots. There is an increase in cytosolic pH and a decrease in apoplastic pH within minutes of gravistimulation. Both changes occur before initiation of a graviresponse. These changes might be

linked to the rapid changes in the membrane potential in *Lepidium* columella cells and to changes in ionic flux around the root cap that have been observed upon gravistimulation. How these changes in pH translate into a directional signal for graviresponse is not clear.

A genetic approach to obtain gravitropic signal transduction mutants has yielded an *Arabidopsis* mutant *arg1* (for altered response to gravity), which is defective in both root and hypocotyl gravitropism, but otherwise is normal in its phenotype. The *ARG1* gene encodes a DnaJ-like protein with a coiled-coil domain at the C-terminal and a membrane-spanning domain in the middle. DnaJ-like proteins are encoded by large gene families in all of the species examined, where they are reported to be involved in protein folding, protein trafficking, and facilitation of multiple signal transduction pathways. Some of these proteins can form large heterooligomeric complexes. The coiled-coil domain is similar to that found in a number of proteins that bind cytoskeletal elements. In *Arabidopsis*, at least three members of this gene family, including *ARG1*, have been isolated. The specific roles of the encoded proteins are still being elucidated, but it is known that expression of *ARG1* transgenically in the *arg1* mutant restores the gravitropic response. Moreover, a suppressor mutation in one of the *ARG1*-like genes almost completely eliminates gravitropism. Taken together, these results strongly argue for an involvement of *ARG1* and related proteins in gravity signal transduction or interpretation of the signal to specific intracellular sites.

4. GRAVITROPIC RESPONSE IS MEDIATED IN PART BY IAA

Whatever the mechanism of signal transduction, at the response site, it is translated into a differential distribution of IAA on the physically upper vs the lower side of the organ, which in turn causes differential growth on the two sides (the Cholodny–Went hypothesis).

Evidence for a mediation by IAA comes from mutants defective in IAA transport and/or response. These mutants are defective in gravitropism in root, shoot, or both. For example, the *AUX1* gene in *Arabidopsis* encodes an auxin influx carrier. The gene is expressed specifically in roots, and *aux1* mutants are agravitropic in roots. The mutants, however, are restored to a wild-type gravitropic response if the roots are exposed to the synthetic auxin, 1-naphthaleneacetic acid (NAA), which, unlike IAA, does not require a carrier-mediated uptake (see Fig. 13-12, Chapter 13).

Many auxin-resistant mutants that are agravitropic show pleiotropic effects, and it is unclear whether the agravitropism displayed by these mutants is not a secondary effect of some other abnormality. *eir1/pin2/agr1* Mutants, however, show agravitropism in roots only. The *EIR1/PIN2/AGR1* locus encodes an auxin efflux carrier and provides genetic evidence for the role of IAA transport in gravitropism in roots, (see Chapter 13). Homologous genes encode auxin efflux carriers that regulate polar auxin transport in shoots (e.g., *PIN1*). Mutations in these latter genes cause an impaired gravitropic response in shoots.

Also, inhibitors of polar auxin transport (e.g., triiodobenzoic acid, naphthylphthalamic acid) or auxin action inhibitors (e.g., *p*-chlorophenoxy isobutyric acid), if applied in the path of polar auxin transport in roots and shoots, inhibit gravitropic curvature from developing.

Finally, some genes, which are specifically induced by auxin (e.g., *SAUR* and *Aux/IAA* genes), are expressed asymmetrically in gravitropic or phototropic curvatures (see Section III).

Measurements of IAA concentration on the two sides of a horizontally placed root or shoot indicate that there is more IAA on the lower than on the upper side. The “fountain” model of IAA transport in roots predicts that in vertically oriented roots, IAA is transported acropetally in the vascular cylinder (probably phloem tissue) toward the root tip. It is also redirected upward from the tip and is translocated basipetally in a polar fashion in epidermal and cortical cells (see Fig. 13-10, Chapter 13). In stems, IAA is translocated from its site of synthesis in shoot tips toward the root in the vascular cylinder. It is also translocated basipetally in a polar fashion in starch-containing sheath cells that comprise the stem endodermis.

Polar transport involves auxin efflux carriers, which are selectively located on the plasma membrane of transporting cells along their basipetal walls. For IAA content to show a net increase on the physically lower side, when the organ is tilted or placed horizontally, a preferential redirection of IAA from the root or shoot tip—more toward the physically lower side and less toward the upper side—must occur (Fig. 27-25). How this is brought about is still unclear. The efflux carriers are located on the basal as well as the lateral walls of transporting cells, and it is possible that there is a selective turning down of basipetal transporters in favor of lateral transporters. Alternatively, there may be a recycling of efflux carriers between basal and lateral walls. Available data are insufficient to discriminate among these possibilities. In shoots, a similar lateral redistribution of IAA between the endodermal cells and from the endodermal to the cortical and epidermal cells is likely to occur.

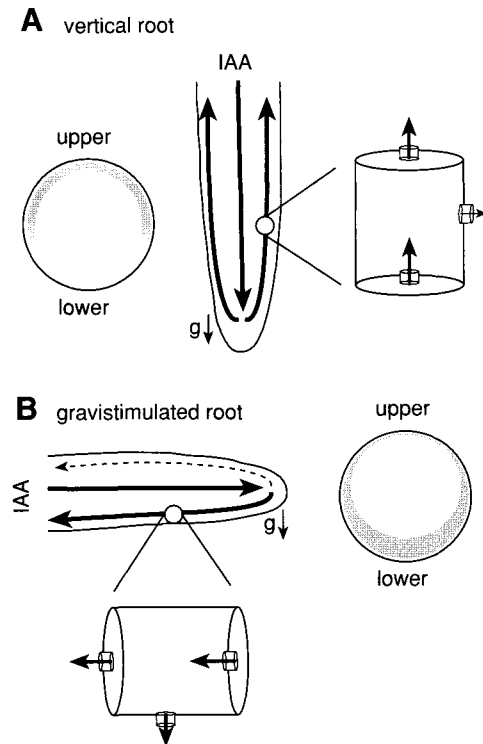


FIGURE 27-25 Model showing the distribution of IAA in root. (A) A vertically oriented root shows normal acropetal transport of IAA in vascular tissue and basipetal translocation of IAA in root epidermis and outer cortex. Cross section of the root shows an even distribution of IAA all around in the epidermis and outer cortex. (B) In a gravistimulated root, the acropetal transport remains undisturbed, but for basipetal transport IAA is redirected preferentially toward the physically lower side, as shown by the solid arrow in the longitudinal view and by the density of shading in the cross-sectional view. Drawings of two cortical cells, one in a vertical and the other in a horizontally placed root, show the distribution of efflux carriers along the lateral and basal walls. Modified from Chen *et al.* (1999).

In roots, however, there is a different problem. The signal is perceived in the columella cells of the root cap, but the response is affected proximally in cells of the elongation zone. The *EIR1/PIN2/AGR1* gene is expressed, and the protein is immunolocalized in cells extending from the quiescent center in the root apex proximally to the elongation zone. It is not expressed in columella cells of the root cap where the signal is perceived. Thus, there must be some means of transmitting the signal across the root apex and the meristematic zone to cells distal to the elongation zone. It is possible that some still unidentified transporter proteins, perhaps encoded by other genes in the *EIR1/PIN2/AGR1* gene family, are expressed in the columella cells and that an auxin gradient actually starts in the columella. In support of

such an assumption, one other member of this gene family has been shown to be expressed in the root cap.

If IAA concentrations are increased on the lower, relative to the upper, side in both horizontally placed roots and shoots, how is it that a root bends toward gravity, whereas a stem curves away from it? The reason is that the threshold IAA concentrations for stem and root growth are different, concentrations of IAA that promote stem growth inhibit root growth (shoot growth requires micromolar concentrations of IAA, but root growth is inhibited at all IAA concentrations above $10^{-8}M$, see Fig. 14-14A, Chapter 14). Hence opposite effects are seen in roots and stems. In roots, the lower side is inhibited from growth, while the upper side grows, resulting in a downward bending of the root. In stems, the opposite occurs, the lower

side grows with concomitant bending of the axis upward.

4.1. Positive Gravitropism in Shoot Structures

In peanut (*Arachis hypogea*), flowers are borne aeri-ally, but after fertilization, gynophore, a specialized structure that bears the ovary (and hence the fruit), grows toward the soil carrying the immature seeds and fruit at its tip and literally buries them in the soil where the seeds and fruit mature (Fig. 27-26A). The gynophore has a shoot-like anatomy, but shows positive gravitropism like a typical root. The downward curvature in the gynophore is mediated by the

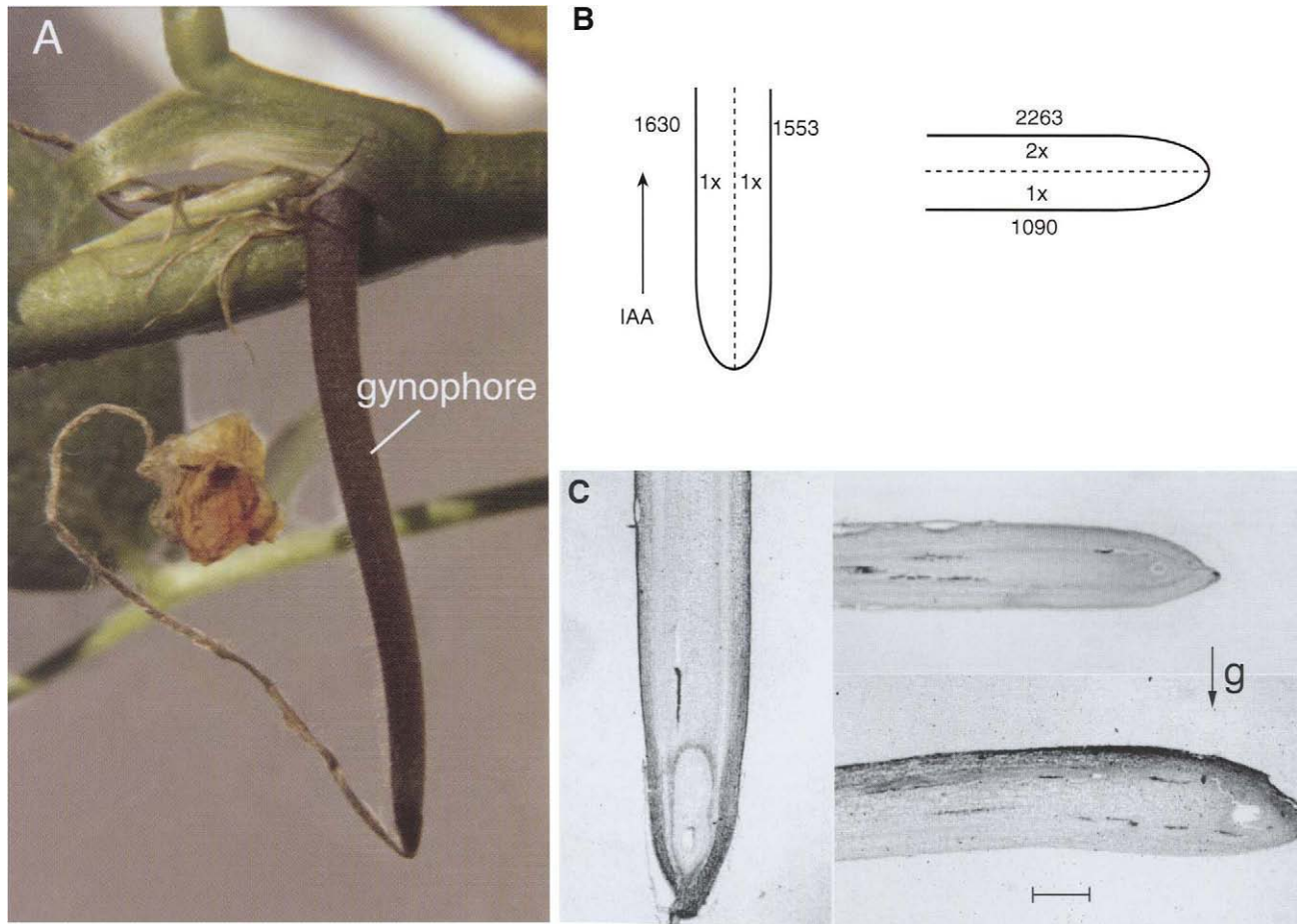


FIGURE 27-26 Peanut gynophore and IAA content. (A) Part of a peanut plant showing a gynophore growing downward toward the soil carrying the immature seeds. Note the wilted flower at the tip. Courtesy of Edgar Moctezuma, USDA-ARS, Beltsville, MD. (B) Distribution of $[^3H]IAA$ in vertically oriented and horizontally placed gynophores. Numbers represent counts per minute. (C) Immunolocalization of IAA in vertically oriented (left) and horizontally placed gynophores at 20 min (top) and 90 min (bottom) after gravistimulation. IAA signal is reflected by the density of immunostaining and, after 90 min gravistimulation, most of it is located on the upper surface. Bar: 0.7 mm. From Moctezuma and Feldman (1999).

differential distribution of IAA. In carefully controlled experiments, it was found that gynophores fed [^3H]IAA, then excised, and placed horizontally show a redistribution of radiolabel; the upper side had about a two fold higher ^3H content than the lower side (in an intact system, the upper side would be growing faster than the lower side during curvature formation). Strictly speaking, ^3H content is not a true indicator of IAA content because of possible IAA metabolism. Therefore, IAA-specific monoclonal antibodies were used to show that IAA content is much higher in the epidermis and cortical cells on the upper side than on the lower side of a horizontally placed gynophore (Figs. 27-26B and 26C). This is the first published use of an IAA-specific antibody, although it should be noted that the antibodies did bind to IAA conjugates to a lesser extent.

4.2. IAA May Not Be the Sole Effector of Gravitropic Response in Roots

Although the evidence for IAA acting as the agent for asymmetric growth in gravitropic response is very strong, there are some indications that it may not be the exclusive or the only signal for asymmetric growth

in roots. Video imaging of maize roots marked with beads and gravistimulated indicates that the response is quite complex and that the Cholodny-Went hypothesis (see earlier discussion) may be too simplistic. In vertically oriented roots, most elongation growth occurs in a zone of cells referred to as the central elongation zone (CEZ). A zone distal to CEZ, referred to as the distal elongation zone (DEZ), consists of cells that normally do not elongate much unless gravistimulated. The first thing that happens on gravistimulation of maize roots is that there is a cessation of growth on both the lower and the upper sides. This is followed by a dramatic elongation of cells in the DEZ on the upper side, whereas cells on the lower side stay arrested. As a result of this growth, a curvature develops (Fig. 27-27). The curvature may overshoot the 90° mark and may be followed by the growth of DEZ cells on the lower side. After back-and-forth oscillations of the root tip, the magnitude of which diminishes with time, the tip finds its correct orientation. This is followed by the growth of cells in the CEZ, whereas cells in the DEZ pass basipetally into the CEZ and eventually into maturity. The existence of these two zones seems to be a common feature in plant roots. What is surprising and difficult to explain

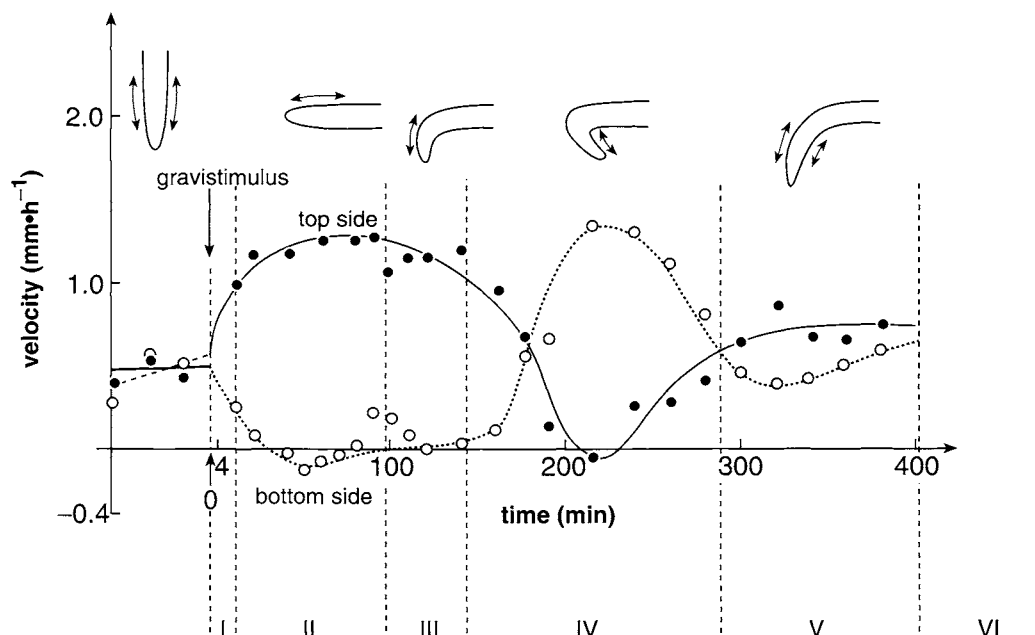


FIGURE 27-27 Time-dependent variation in growth rate (velocity, mm h^{-1}) on the upper (top) and lower (bottom) sides of a gravistimulated maize root. On gravistimulation, the top side grows faster at first, then the rate slows down and may even become negative, while the bottom side begins to grow. After a back and forth oscillation, the root grows vertically downwards. The curvature response is divided arbitrarily into six phases indicated I through VI. From Ishikawa *et al.* (1991).

is that the growth of cells in the DEZ, which causes curvature, seems to be independent of auxin. It is insensitive to exogenous auxin to concentrations that completely inhibit root growth. In contrast, cells in CEZ are sensitive to auxin, which means that DEZ cells, as they pass basipetally into CEZ with root growth, acquire sensitivity to auxin.

Among other effectors of gravitropic curvature, calcium gradients, pH gradients, and electrical fields have all been implicated. Gravistimulation is reported to cause a rapid induction of polar calcium movement toward the lower side of the root cap in maize roots. Calcium is a known inhibitor of growth because it binds to pectins in the cell wall. Hence, it is conceivable that an enhanced calcium concentration on the lower side in the DEZ induces curvature. Application of calcium chelators, such as EDTA or EGTA, inhibits curvature development in maize roots, probably because growth is not arrested on any side. Enhanced acidity on the upper surface of gravistimulated maize roots has also been reported, which is consistent with enhanced wall loosening on the upper side. Electrical fields have been reported to affect cells in the CEZ and DEZ in an opposite manner. In mung bean (*Vigna mungo*) roots, in the CEZ, curvature was driven by the inhibition of elongation, whereas curvature in the

DEZ was primarily due to the stimulation of elongation.

5. INTERACTION BETWEEN LIGHT AND GRAVITROPISM

The interaction of light and gravity and the involvement of hormones in such an interaction are little studied fields, but such interaction is known to occur and affect the orientation of root and shoot growth. A dramatic effect of light on graviresponse of roots is shown using wild-type and starchless mutant seedlings of *Arabidopsis*. If these seedlings are irradiated with unilateral white light in a gravitational field, the roots of wild type grow at an angle intermediate between 0 and 90°. In contrast, the roots of starchless mutants grow at angles closer to 90° (Fig. 27-28A). If the seedlings are irradiated from below, the roots of starchless mutants may show a 180° turn and grow vertically upward, whereas the roots of wild-type seedlings grow away from the light but still remain below the horizontal (Fig. 27-28B).

In their normal growth, of course, most roots are not exposed to direct light; however, in some cases, root gravitropism is known to be modulated by light. The

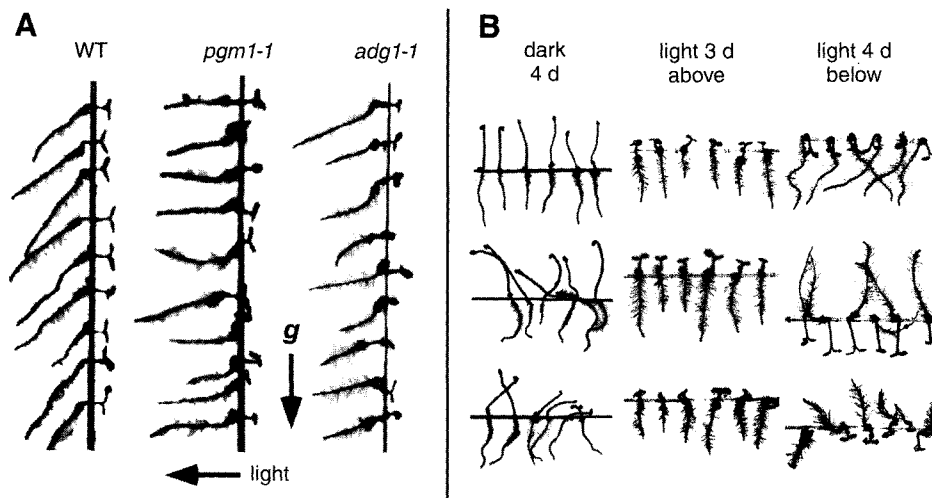


FIGURE 27-28 Negative phototropism of roots in wild-type and two starchless mutants of *Arabidopsis*. (A) Irradiation with unilateral light. Seedlings were germinated and grown for 3 days in unilateral white light. The roots of starchless mutants display much greater negative phototropism than wild-type roots. (B) Irradiation with light from above or below. Seedlings of wild type (top row), *pgm 1-1* (middle row), and *adg 1-1* (bottom row) mutants were germinated and grown in continuous darkness or in light from either above or below. Dark-grown mutant roots deviated more from the vertical than those of the wild type. Grown in light from above, roots of all three genotypes grew uniformly toward gravity. However, light from below caused the mutant roots to grow away from the light and above the horizontal, whereas wild-type roots grew partly away from the light but still remained below the horizontal. The hypocotyls of light-grown seedlings in each case were positively phototropic. Seedlings are shown in outline form, drawn from digitized photographs. From Vitha *et al.* (2000).

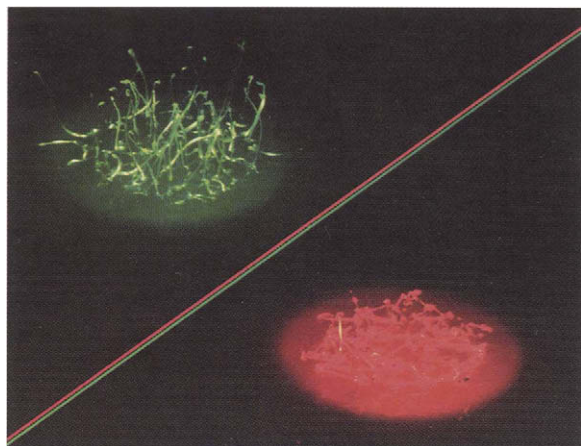


FIGURE 27-29 Growth of *Arabidopsis* seedlings in green light (top left) and in red light (bottom right). In red light, seedlings lose their ability to respond to the gravitropic vector and grow in every direction. From Liscum and Hangarter (1993).

best studied example is provided by the Merit cv of maize. In this plant, roots grow horizontally in dark, but if irradiated with a pulse of red light, they begin to grow downward within 1 h with the response completed in 2–3 h. Since the response is red/far-red reversible, it is mediated by phytochrome. Similar responses are seen in other roots.

In shoots, phytochrome is reported to both enhance and decrease graviresponsiveness. Seedling shoots of most plants show negative gravitropism, i.e., grow upward, when grown in darkness. *Arabidopsis* seedlings grown under red light, however, lose their ability to orient themselves to the gravity vector (Fig. 27-29). This loss is reversible by FR and is controlled by both phyA and phyB. “Lazy” mutants are known in several plants (e.g., maize, tomato, rice). In maize and tomato, shoots of mutant plants grow downward in response to irradiation with a low fluence R light, a response that is reversible by a subsequent FR pulse. Tomato has several phytochrome genes. For the *lazy-2* mutant of tomato, the involvement of at least phyA and phyB1 has been demonstrated.

6. SECTION SUMMARY

Plants perceive gravity in specialized cells, called statocytes, which occur in central columella of root caps and in bundle sheath or starch-sheath cells in shoots. Amyloplasts appear to be the main statoliths in statocytes of vascular plants. When a root or shoot is tilted from its normal vertical position, the amyloplasts are displaced from their position of equilibrium.

This change in signal (probably pressure) is sensed by the underlying system of membranes and cytoskeleton composed of endoplasmic reticulum, plasma membrane, and microtubules and microfilaments. The details of such a perception mechanism are unknown. Also unknown are the steps by which the change in the gravity signal is transmitted to the site of response, although they probably involve secondary messengers, such as Ca^{2+} and Ca^{2+} /calmodulin-dependent processes in a cell. Some mutants in gravitropic signaling have been identified, and rapid progress is expected in this interesting area. Asymmetric growth at the response site is thought to be due to a lateral redistribution of IAA in a gradient—high at the physically lower side and low at the upper side of a horizontally placed root or shoot. However, no connecting links between signal perception/transduction and lateral redistribution of IAA have been found yet. In roots, the situation is complicated because the site of asymmetric growth is removed from the site of gravity perception. Moreover, there is a component of graviresponse in roots that appears to be independent of IAA and that may be mediated by changes in Ca^{2+} levels. Nonetheless, IAA seems to be the principal agent mediating asymmetric growth. How IAA brings about asymmetric growth and the role of other hormones, especially ethylene, in this process are covered in the following section. Light acting via phytochrome is known to modulate the graviresponse of roots and shoots, but the details of such interaction, including roles of hormones, need elucidation.

SECTION III. ASYMMETRIC GROWTH DURING PHOTO- AND GRAVITROPISM

1. IAA CONTENT AND WALL EXTENSIBILITY

As shown elsewhere, in axial organs, epidermal cells with their cross-polylamellate outer tangential walls exercise a controlling effect on the rate of elongation growth. Furthermore, one of the major effects of IAA on elongation growth is wall loosening and enhancement in the plastic extensibility of cell walls (see Chapter 15). Quantitative changes in IAA content in epidermal cells after tropic stimulation are not available, but the red light-induced inhibition of straight growth in maize coleoptile involves a selective diminution in the IAA content in epidermal cells by metabolism and/or transport (see Table 26-8). Thus, it is possible that as a result of phototropic induction,

there is a decrease in IAA content in epidermal cells on the irradiated side, which in turn causes a diminution in wall extensibility and a reduction in growth on that side. Simultaneously, a corresponding increase in the IAA content in epidermal cells on the shaded side causes enhanced wall extensibility and increased growth on that side. Similar changes in IAA content in epidermal cells can be visualized during gravitropic curvature.

Correlated with these changes in wall extensibility, the orientation of cortical microtubules (MTs) along the outer tangential walls of epidermal cells also changes. These changes have been studied mostly in shoot tissues. In negatively gravitropic stems/coleoptiles, during curvature, the MT orientation along the outer tangential wall of epidermal cells on the physically upper side, which shows less growth, changes from being transverse to being predominantly longitudinal. On the lower side, which grows correspondingly more, the orientation remains transverse (Fig. 27-30). Similarly, in phototropism, the faster growing shaded side retains transversely oriented MTs along the outer tangential wall of the epidermal cells, whereas on the irradiated side, the MT orientation changes from being transverse to longitudinal. The causality, whether the auxin concentration determines MT orientation or whether plasma membrane-related changes or changes in the mechanical tissue strain determine the MT orientation, remains uncertain (for a discussion of MT orientation during straight growth, see Chapter 15).

Similarly, in grass pulvini, on gravistimulation, cells on the lower side of the pulvinus elongate, whereas cells on the upper side remain arrested in growth. A differential distribution of IAA in the cells on the upper and lower side has been reported. Moreover, in a detailed study on maize stem pulvinus, it was shown that cortical microtubules in pulvinar cells remain transversely oriented (to permit elongation growth) and do not change to random or longitudinal until after the pulvinus loses its capacity to gravire-spond.

1.1. Auxin-Induced Gene Expression and Asymmetric Growth

A convincing proof that IAA mediates asymmetric growth is provided by one of the loci identified in a screen for phototropic mutants. The *NPH4* locus in *Arabidopsis*, as judged from mutational analysis, affects phototropism at both low and high fluences of light. It also affects several other physiological responses, including gravitropism. The *NPH4* gene encodes an auxin response factor, ARF7. Auxin response factors,

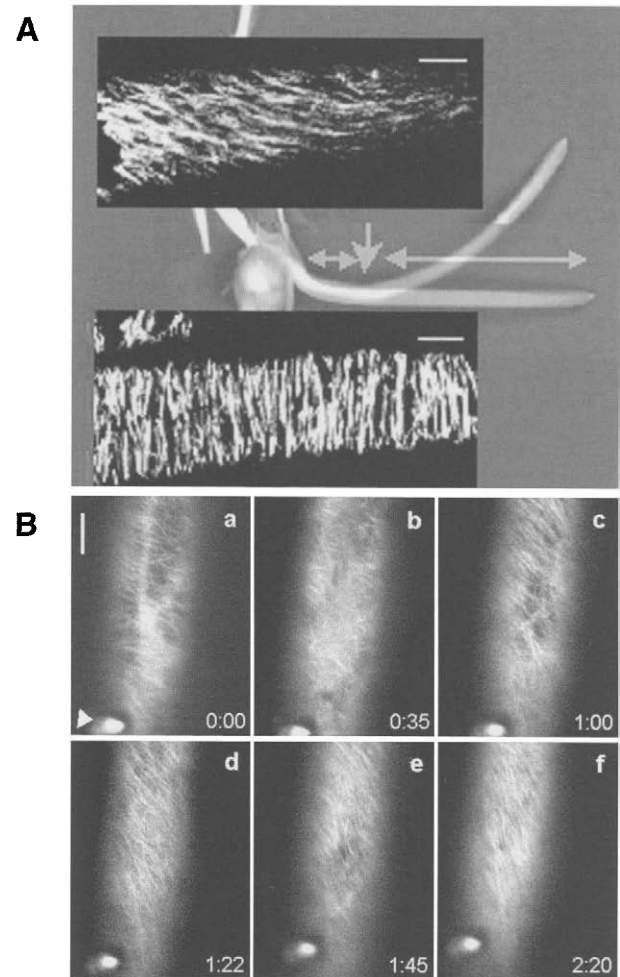


FIGURE 27-30 Reorientation of cortical microtubules (MTs) in the outer epidermis during gravitropic bending. (A) A maize (*Zea mays*) seedling (grown for 3 days under weak red light) has been tilted by 90°. The image, a double exposure, shows gravitropic bending 2 after the onset of stimulation. Double arrows indicate parts not bending. The single arrow points to the site of bending in the seedling. Cortical microtubules have been visualized by immunofluorescence in the upper and lower flanks of the coleoptile. Note the longitudinally oriented microtubule array in the upper flank and the transversely oriented array in the lower flank. Size bar: 10 μm . (Courtesy of Peter Nick, University of Freiburg, Freiburg, Germany). (B) *In vivo* immunofluorescence of MTs in maize coleoptiles during gravistimulation. The orientation of MTs along the outer tangential wall of epidermal cells on the upper side changes from horizontal to longitudinal as growth slows along that surface. The same region of this cell was imaged at different time intervals in hours (right bottom corner of each image). Bar at top left in (a) = 5 μm . From Himmelspach *et al.* (1999).

as explained in Chapter 22, act as transcriptional activators. They bind DNA and may dimerize with Aux/IAA proteins or other ARFs to regulate auxin-induced gene expression in a tissue-/organ-specific manner. In conformity with that role, the *nph4* mutant shows a severe down regulation in the expression of several

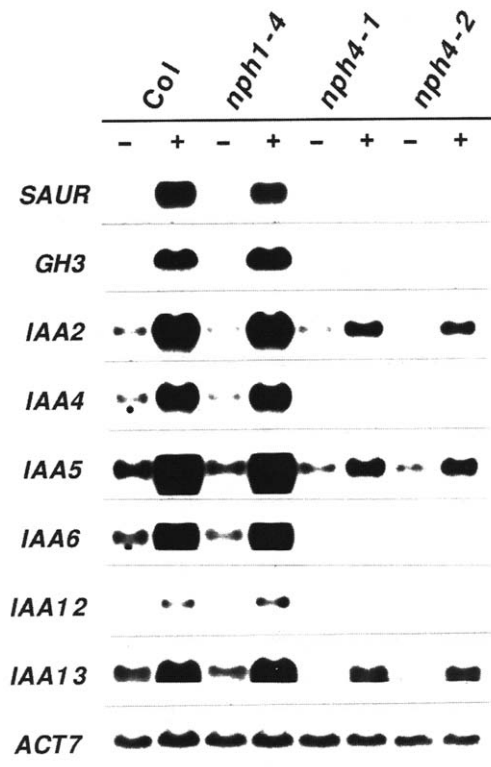


FIGURE 27-31 Expression of auxin-induced genes in wild-type (Columbia, Col) and *nph4* seedlings of *Arabidopsis*. Total RNA was extracted from seedlings exposed to 100 μ M IAA (+) or solvent (–) for 1 h. Samples (20 μ g each) were separated on gels and hybridized to 32 P-labeled gene-specific probes. *SAUR*, *GH3*, and *IAA2*–*IAA13* are early auxin-induced genes (see Chapter 22). The *ACTIN7* (*ACT7*) probe was used as a RNA loading control. RNA from *nph1-4* was used as an additional positive control. From Stowe-Evans *et al.* (1998).

Aux/IAA genes, as well as *SAUR* and *GH3* genes (Fig. 27-31).

Aux/IAA and *SAUR* genes, as discussed in Chapter 22, are primary auxin responsive genes expressed within minutes of auxin treatment. Both types of genes are reported to show a differential expression on the two sides of a photo- or gravitropically stimulated organ. For example, mRNAs of *SAUR* genes are localized in epidermal and cortical cells. During asymmetric growth of shoots undergoing negative gravitropism, or phototropism, the transcripts accumulate preferentially in the more rapidly growing side. As the curvature is completed, the distribution of transcripts becomes normal again (Fig. 27-32). An *Aux/IAA* gene in pea and another in cucumber are reported to be expressed differentially on the two sides of a gravi-stimulated organ. Unfortunately, the precise roles of these genes and the nature of their encoded products are still unclear, and their connection with asymmetric growth is unknown. Gene-specific mutations and/or

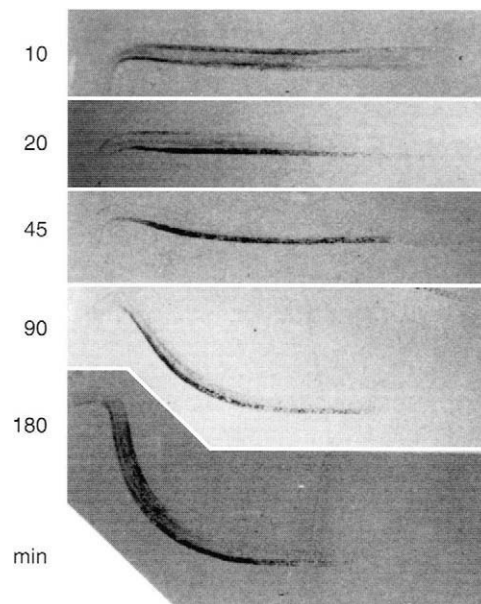


FIGURE 27-32 Tissue print localization of *SAUR* transcripts in hypocotyls of etiolated soybean seedlings. Hypocotyls were sampled after gravistimulation for indicated times (left). They were sectioned longitudinally, and cut surfaces were blotted onto nylon membranes and hybridized with the *SAUR* antisense 35 S-labeled RNA probe. The shoot tip is to the left in each autoradiogram. Autoradiograms show that the mRNA for *SAUR* genes is localized primarily in the cortical and epidermal cells. In vertically oriented hypocotyls [represented here by the topmost autoradiogram (10 min)], the transcripts are distributed evenly on all sides. With increasing time to 90 min, the transcripts become progressively more localized toward the faster-growing, physically lower side. By 180 min, as the apical part of hypocotyl assumes a more vertical position, the distribution of transcripts becomes more normal. From Guilfoyle *et al.* (1990).

gene knockout techniques may provide useful clues about the roles of these genes in asymmetric growth.

The other target genes that may be associated with differential growth also remain elusive. Genes encoding proteins that are likely to be involved in cell growth, such as expansins, XETs, endo-1, 4- β -glucanases, water channel proteins, and tubulin, have been identified in elongating tissues after auxin, GA, or brassinosteroid treatment (see the section on cell growth in Chapter 15), but the participation of these genes in asymmetric growth remains to be seen. In a growing organ, most of these genes are probably already induced and a differential expression of these genes may be difficult to demonstrate.

It will be recalled that phototropic curvature by low fluence blue light is enhanced by red light and that phytochrome mediates that response (see Fig. 27-5). It has been shown that the enhancement response is mediated by phyA acting in a low fluence R/FR reversible mode and, further, that it involves participa-

tion by the NPH4/ARF7 transcription activator. It is of interest in this connection that *nph4* mutants fail to exhibit phototropism, especially phyA-dependent enhancement.

1.2. Auxin/Ethylene Interactions in Asymmetric Growth

Although auxin seems to be the primary mediator of asymmetric growth in gravi- and phototropic curvatures, other hormones, especially ethylene, are known to interact in such growth. For example, shoot gravitropism is often accompanied by a large ethylene production. The critical evidence, however, comes from genetic data. Many mutants isolated in screens for ethylene response have turned out to be auxin homeostasis or auxin transport mutants (e.g., *hls1*, *eir1*); many auxin transport and/or response mutants (e.g., *aux1*, *axr1*, *axr2*, *axr3*) also show resistance to ethylene.

In some studies, ethylene treatment has been shown to modulate auxin-induced gravi- or phototropic curvature. The *diageotropica* (*dgt*) mutant of tomato shows pleiotropic defects, which are typically seen in auxin-deficient or -insensitive mutants. Mature plants grow prostrate, lack lateral roots, have shortened internodes, lack apical dominance, and show retarded vascular development. The mutant tissues do not produce ethylene when cut segments are treated with auxin, and the expression of some, not all, auxin responsive genes, such as *SAUR* genes, is severely curtailed. However, the mutant has normal auxin content and IAA is translocated in hypocotyls and roots at rates similar to those in the wild-type plant. Hence, the mutant is thought to be defective in auxin perception or response. However, ethylene interacts with auxin signaling in some still unknown manner. Inhibitors of ethylene action reduce wild-type gravicurvature, which indicates participation by ethylene in the formation of curvature, and extremely low (0.0005–0.001 $\mu\text{l/liter}$) ethylene concentrations can restore the gravitropic response of the *dgt* mutant to wild-type levels. Higher concentrations of ethylene, however, inhibit the gravitropic response of wild type as well as the *dgt* mutant.

nph4 Mutants of *Arabidopsis* do not respond to exogenous auxin, but in the presence of 50 $\mu\text{l/liter}$ ethylene, they show more or less a normal phototropic curvature (Fig. 27-33). Also, *nph4* seedlings grown in darkness in air lack an apical hook, but if grown in ethylene they develop an exaggerated hook. These ethylene effects are not due to a lack of ethylene production in *nph4*; mutant seedlings produce the same amount of ethylene as the wild type. How ethylene affects these phenotypic changes in *nph4* is not known,

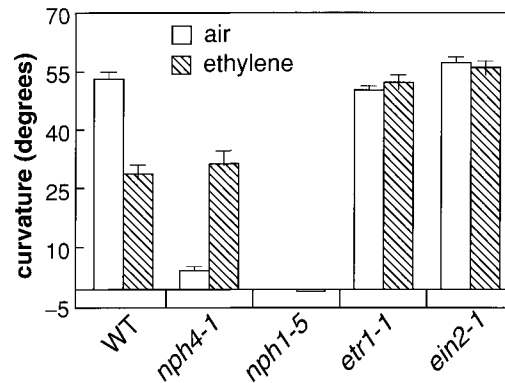


FIGURE 27-33 Effect of ethylene on phototropism of wild-type (Col) and *nph4* seedlings. Phototropic curvature of 3-day-old seedlings grown in darkness under ambient air conditions (open bars) or exposed to 50 $\mu\text{l/liter}$ ethylene (hatched bars) and then exposed to 8 h of unilateral blue light ($0.1 \mu\text{mol m}^{-2} \text{s}^{-1}$). Note that in the presence of ethylene, the *nph4* mutant shows the wild type response. Data for *nph1* and ethylene-insensitive mutants *etr1* and *ein2* are included as internal controls. In contrast to *nph4*, *nph1* did not show any curvature, whereas *etr1* and *ein2* showed the same degree of curvature in air and in ethylene. Data represent the mean response of at least 40 seedlings from at least two replicate experiments. Error bars indicate SE. From Harper *et al.* (2000).

but it is thought that it enhances the tissues' sensitivity to endogenous auxin. These data also imply that NPH4 acts at the crossroads for auxin and ethylene signaling.

It was mentioned earlier that *Arabidopsis* seedlings grown under red light lose their gravitropism. Cytokinin treatment restores these seedlings to their normal gravitropic response, and it is thought that it does so via ethylene synthesis.

Among other hormones, gibberellins and brassinosteroids have been implicated in tropic growth, but how they participate in such growth is not clear.

2. SECTION SUMMARY

IAA seems to mediate the asymmetric growth of roots and shoots by its effect on wall loosening in epidermal (or peripheral) cells, which are thought to regulate the rate of extension growth in axial organs. For example, a decrease in IAA content on the physically upper side in a negatively gravitropic shoot is predicted to result in a reduced wall loosening on that side, resulting in an upward curvature. Concomitant changes in the orientation of cortical microtubules and, by inference, newly laid cellulose fibrils in outer walls of epidermal cells have been recorded. That IAA mediates asymmetric growth is also shown by the identification of NPH4 as one of the loci involved in photo- or gravitropism and that it encodes an auxin response

factor, NPH4/ARF7. ARFs are DNA-binding proteins that dimerize with other ARFs or with Aux/IAA proteins to regulate the expression of other auxin-induced genes. *Aux/IAA* and *SAUR* genes are specifically induced by auxins, and although their roles are unknown, their transcripts accumulate differentially on the two sides of an axial organ showing phototropic or gravitropic bending. Auxin and ethylene interact to regulate many plant responses, including asymmetric growth. Auxin-insensitive mutants, such as *nph4 Arabidopsis* and *diageotropica* tomato, are partially restored to wild type in the presence of ethylene. This suggests the presence of common nodes in auxin and ethylene signaling and that NPH4/ARF7 may be one such node. However, there are as yet no connecting links between the perception of the phototropic or gravitropic signal and the lateral mobilization of IAA or induction of IAA-induced genes involved in asymmetric growth.

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